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13. ABSTRACT *(Maximum 200 Words)*

The basic premise is that the turnover of dopamine (DA) by monoamine oxidase (MAO) places an oxidative stress on the mitochondria of DA neurons. The generation of H_2O_2 by MAO induces formation of glutathione disulfide (GSSG) and protein-glutathione mixed disulfides (PrSSG). Loss of essential protein thiol groups, such as those required by Complex I, compromises mitochondrial electron transport. In turn, mitochondrial dysfunction contributes to the progression of Parkinson's disease and to the damaging effects of environmental neurotoxins. In year 1, we showed that MAO suppressed respiration & electron transport, and elevated mitochondrial PrSSG. Work in 2nd year verified the pivotal role of H_2O_2 and showed for the first time that pyruvate dehydrogenase (PDH) is also detrimentally affected by MAO. And most important, reversal of damage by pyruvate or succinate was accompanied by removal of PrSSG from the inner mitochondrial membrane. These results support the working hypothesis and help to clarify the pathophysiology of neurodegenerative mechanisms affecting DA neurons. Over the longer range, the new leads concerning (a) the thiol redox state of mitochondria and (b) mechanisms that reverse damage, can lead to improved methods to protect DA neurons from environmental neurotoxins and from the ravages of Parkinson's disease.

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TABLE OF CONTENTS

INTRODUCTION

This proposal addresses the general goals of providing improved understanding of the pathophysiology of neurodegenerative processes affecting dopamine-secreting neurons. The research focuses on oxidative stress and damage to mitochondria evoked by the action of monoamine oxidase, and encompasses developing information that could lead to new treatment strategies for delaying or preventing the progression of Parkinson's disease. The proposal deals reversible damage to mitochondrial electron flow and mitochondrial respiration based on the formation of protein-disulfide linkages with oxidized glutathione (viz., protein mixed-disulfides). As described in the original Statement of Work, the proposal, in general, deals with: (1) Monoamine oxidase in the outer membrane of mitochondria within dopamine neurons; (2) Mechanisms of damage by oxidative stress, specifically that associated with role of glutathione and protein thiols in modulating the basic respiratory function of neural mitochondria; (3) Mechanisms for reversal of mitochondrial damage, and; (4) Laying the basic groundwork for new therapeutic strategies to protect against environmental neurotoxins and to prevent further dopaminergic damage in Parkinson's disease. Significant progress was made and/or extended for aspects of goals 1, 2 and 3 during the 2nd year of this grant.

BODY OF REPORT

ABBREVIATIONS USED:

dopamine (DA),
glutathione (GSH),
glutathione disulfide (GSSG)
monoamine oxidase (MAO),
Parkinson's Disease (PD),
protein thiol (PrSH)
protein mixed-disulfide (PrSSG)
pyruvate dehydrogenase (PDH)

OVERVIEW

This project deals with a new finding that the activity of monoamine oxidase (MAO), an outer mitochondrial membrane enzyme, alters electron flow at the inner mitochondrial membrane and suppresses mitochondrial State 3 and State 5 respiration (Cohen, Farooqui, & Kesler, 1997; Cohen and Kesler, 1999).

Electron flow, coupled to consumption of oxygen (respiration), is necessary for the production of ATP. Therefore, an implication is that the natural turnover of the neurotransmitter dopamine (DA) by MAO can affect energy production (ATP formation) and cellular viability. Parkinson's disease (PD) is characterized by an unexplained loss of DA neurons that originate in the substantia nigra, a melanized region of midbrain. It is well established that PD is itself characterized by a defect in mitochondrial respiration that targets Complex 1 activity of mitochondrial electron chain. The defect in Complex 1 is evident from autopsy studies (e.g., Schapira et al., 1990; Schapira, 1999). The same defect characterizes PD evoked by exposure to the environmental toxin MPTP (1-methyl-4-phenyl-2,3,5,6-tetrahydropyridine). The basis for

the defect in idiopathic PD has not yet been established. However, it is believed that both genetic factors (Polymeropoulos et al., 1997; Kitada et al., 1998) and environmental toxins (Gorrell et al., 1996) may be involved.

In our working hypothesis, DA represents an "endogenous" neurotoxin. Its enhanced turnover in PD (Hornykiewicz and Kish, 1986) represents an endogenous oxidative stress evoked by MAO. The basic neurochemistry that underlies susceptibility of mitochondria to oxidative stress is given by the following equations:

$$DA + O_2 \longrightarrow H_2O_2 + NH_3 + 3,4\text{-diOH-phenylacetaldehyde} \quad \text{(Eqn. 1)}$$

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O \quad \text{(Eqn. 2)}$$

$$GSSG + PrSH \longrightarrow PrSSG + GSH \quad \text{(Eqn. 3)}$$

In this sequence, MAO produces hydrogen peroxide (H_2O_2, eqn. 1), which provides a focus for an oxidative stress. The peroxide is removed by the enzyme glutathione (GSH) peroxidase (eqn. 2); glutathione disulfide (GSSG) is formed in the process. The GSSG then reacts with thiol groups of proteins (PrSH, eqn. 3), forming protein mixed-disulfides (PrSSG). Many proteins, including the components of Complex 1, require thiol groups for their enzymatic function. Therefore, loss of essential thiols (PrSH) via formation of PrSSG can lead to loss in enzymatic function. However, the loss is reversible; both the formation and the reversal reactions are enzymatically catalyzed.

Previous research targets for the 1st year of this grant were: (a) To determine if MAO activity suppresses mitochondrial respiration in concordance with the suppression of electron flow, (b) to determine if PrSSG levels rose simultaneously, and (c) to evaluate the actions of selective MAO-A and MAO-B substrates and inhibitors. Progress was made and these goals were met during the first year. Targets for the 2nd year emerged, in part, from the prior studies and, in part, from information appearing in the literature. The new targets included (1) delineating the special role of MAO-generated H_2O2 (as opposed to other products of the MAO reaction), (2) distinguishing between effects on mitochondrial Complex I vs. the pyruvate dehydrogenase (PDH) complex, and (3) assessing the presence and mechanisms for mitochondrial repair during electron flow. As described below, significant progress was made in each of these areas. A manuscript concerning the differing effects of MAO on PDH and Complex I is currently in review for publication (Cohen & Kesler, in review).

EXPERIMENTAL STUDIES:

**** Note: Figures & figure legends can be found in the rear**

1. Pinpointing the role for MAO-generated H_2O_2:

A major premise underlying this research program is that H_2O_2 generated by MAO evokes mitochondrial damage. However, as shown in Eqn. 1, there are two additional products of the MAO reaction: an aldehyde and ammonia. Recent reports have indicated that aldehydes derived from monoamines might also exert toxicity. Therefore, it is important to verify the role of H_2O_2 in mitochondrial toxicity.

We took several approaches to verifying the role of H_2O_2. First, the enzyme catalase was used to scavenge H_2O_2 generated by MAO at the outer mitochondrial membrane. As shown in Fig. 1, catalase (10 μg/mL) was almost fully protective and prevented damage to mitochondrial electron flow. This result links damage by MAO specifically to the generation of H_2O_2.

Second, azide was used to inhibit endogenous catalase. Although brain possesses only small amounts of catalase, mainly in astrocytes, glia, and catecholamine neurons, the presence of microperoxisomes in mitochondrial preparations provides a means of suppressing damage to mitochondria. As shown in Fig. 2, azide potentiated damage by 500 μM DA at 20, 40, & 60 minutes. This result indicates that endogenous catalase present in mitochondrial preparations can suppress damage. It should be noted that previous investigators (Berridge & Tan, 1993) had shown that azide does not itself inhibit electron flow measured by dye reduction with MTT because the block at cytochrome oxidase is too far down the electron transport chain to affect the electron flow assay. Azide also potentiated damage over the concentration range 100-500 μM DA (Fig.3). The results verify the importance of H_2O_2 in MAO-mediated damage.

Lastly, a completely different H_2O_2-generating system, consisting of glucose plus glucose oxidase, was substituted for MAO activity. Glucose oxidase generates H_2O_2, but not an aldehyde nor ammonia. As shown in Fig.4, glucose oxidase substituted efficiently for MAO and inhibited mitochondrial electron flow, with potentiation by azide. Therefore, the overriding importance of H_2O_2, as opposed to formation of an aldehyde or ammonia (Eqn. 1), is confirmed.

Conclusion: These studies firmly establish that an H_2O_2-scavenging enzyme, catalase, suppresses damage, while an inhibitor of endogenous trace amounts of catalase (azide) potentiates damage. Moreover, an independent H_2O_2-generating enzyme, glucose oxidase, can be substituted for MAO. Therefore, damage by MAO is linked specifically to H_2O_2 and the additional enzymatic products (3,4-dihydroxyphenylacetaldehyde and ammonia) can be excluded as contributing in any significant way to mitochondrial damage. These results uphold and support a major premise of the research program.

2. Inhibition of pyruvate dehydrogenase by MAO.

The enzyme complex pyruvate dehydrogenase (PDH) generates NADH (Eq. 4), which, in turn, initiates electron flow in the mitochondrial respiratory chain. PDH is localized to the matrix side of the inner mitochondrial membrane. Electron flow in the inner membrane can be initiated experimentally by pyruvate via the reduction of ubiquinone (coenzyme Q), catalyzed by Complex I (NADH-CoQ reductase) (Eq. 5), or by succinate via Complex II (succinate-CoQ reductase).

$$\text{Pyruvate} + \text{CoA} + \text{NAD} \rightarrow \text{acetyl CoA} + CO_2 + \text{NADH} \qquad \text{(Eqn. 4)}$$

$$\text{NADH} + H^+ + \text{CoQ} \rightarrow NAD^+ + CoQH_2 \qquad \text{(Eqn. 5)}$$

Both PDH (Ali et al., 1993) and Complex I (Gutman et al., 1970) are sulfhydryl-dependent enzyme complexes and, therefore, they can be inhibited by loss of essential thiol

groups during an oxidative stress, such as that associated with production of H_2O_2. The inhibition of mitochondrial electron flow by MAO is substantially greater when pyruvate is used as substrate compared to succinate (Cohen et al., 1997). The latter observation implies that the defect is localized to Complex I. In contrast, electrons derived from the metabolism of succinate flow to coenzyme Q via Complex II, bypassing Complex I. However, since both PDH and Complex I exhibit a dependence on protein thiols, it is conceivable that the defect in pyruvate-based metabolism may be localized to PDH, rather than Complex I. Complex I has been extensively studied. It is suppressed by 30-40% in autopsy specimens from patients with Parkinson's disease (Schapira et al., 1990); brain Complex I is similarly suppressed by the neurotoxin MPTP. Therefore, experiments were constructed to test for the possible involvement of PDH.

The results show that damage to PDH is delayed in time and, therefore, suppression of respiration is a primary event. Nonetheless, inhibition of PDH activity does occur, raising a new possibility that defects in both Complex I and PDH can contribute to mitochondrial malfunction in Parkinson's disease or in the response to neurotoxins.

Our experimental results in the study of PDH are as follows: Earlier experiments with rat brain mitochondrial utilized an exposure time of 15 minutes at 27°C to evaluate the effect of tyramine on mitochondrial respiration. For respiratory studies, exposure times must be brief because isolated mitochondrial show a fall off in respiratory function with time. The current experiments were conducted under the same conditions. The results in Fig. 5 show that mitochondrial respiration was diminished (as previously reported). After 15 min exposure to 500 μM tyramine (a mixed MAO-A/MAO-B substrate), respiratory inhibition was 3.4-fold greater than the apparent suppression of PDH. However, the small change in PDH did not achieve statistical significance ($p=0.071$). Therefore suppression of respiration in our experiments is a primary event and does not follow secondarily from any change in PDH. And, therefore, early suppression of respiration is best attributed to damage downstream at Complex I.

Fig. 6 shows that inhibition of PDH occurs progressively after longer exposure to H_2O_2-generated MAO (that is, the damage is delayed in time). Damage to PDH studied after 30 min was completely blocked by inhibition of MAO (Fig. 7). Therefore, damage was mediated by eqns. 1-3.

Conclusions: We conclude that although PDH and Complex I are both targets for MAO-mediated damage, Complex I is more susceptible. In Parkinson's disease, surviving DA neurons exhibit increased turnover of DA associated with increased metabolism by MAO (Hornykiewicz & Kish, 1986). Therefore, the current observations may reflect on the known brain defect in Complex I in Parkinson's disease and on the origin of the mitochondrial lesion. PDH is inhibited on longer exposure and may also play in role in expression of Parkinson's disease or in the response to environmental toxins. Based on this new finding concerning PDH, we plan to study autopsy specimens from Parkinson brain for possible changes in PDH; apparently this enzyme was completely overlooked in the earlier studies by others on autopsy specimens.

3. Repair of damage to mitochondrial electron flow is associated directly with removal of PrSSG via the metabolism of pyruvate or succinate.

A basic premise of this research program is that damage to mitochondria by MAO is based on the formation of PrSSG (protein-GSH mixed disulfides; Eqns. 1-3). with essential thiol groups of Complex I (or, as now shown above, perhaps PDH, as well). A 10-fold rise in PrSSG was documented during exposure of mitochondria to DA or tyramine (Progress report for year 1 and Cohen & Kesler, 1999). Therefore, it was important to determine whether the reversal of functional damage to mitochondrial during the metabolism of pyruvate or succinate was dependent upon the removal (reversal) of PrSSG.

Initial experiments were quite disappointing. Reversal of PrSSG formation proved very small and non-significant when measured in intact mitochondria. However, we reasoned that reversal of damage would be initiated at the inner membrane, which is the site of metabolism of pyruvate or succinate. Therefore, reversal of PrSSG would be initiated at the inner membrane, which might undergo substantive change, before reducing equivalents reached the intermembrane or matrix spaces, or the outer membrane. The outer membrane, in particular, because it is the site for MAO, might undergo relatively greater chemical changes initially (formation of PrSSG during MAO activity). When reversal of PrSSG is studied with whole mitochondria, the contribution from the inner membrane might be too small to contribute significantly to the total PrSSG (outer & inner membranes, plus intermembrane and matrix spaces).

To address this experimental problem, we elected to isolate the inner (or mitoplast) membrane in order to study the change in PrSSG selectively at this site. The membrane encompasses the entire electron transport chain, and specifically includes Complex I. The inner membrane was isolated by the method described by Sottocasa et al., 1967. The inner membrane was obtained free of matrix and intermembrane space proteins, and substantially free of the outer membrane.

Preliminary results from these experiments are shown in Fig. 8. Pyruvate/malate (10 mM each) was used as mitochondrial substrate. Fig. 8 (left side) shows that electron flow was restored by about 50% after 15-min exposure to pyruvate/malate at 27°C when tyramine had been used as substrate for MAO. Damage by DA was also reversed, but to a lesser extent.

The right side of Fig. 8 shows that PrSSG formation was simultaneously reversed by 50% by pyruvate/malate. This result indicates that reversal of PrSSG and reversal of damage are linked. In terms of quantitation, it is not necessary for reversal of each to be exactly the same. The reason is that we do not know which proteins have undergone selective reversal of PrSSG in the inner membrane. Various proteins (including Complex I) may undergo reversal, with restoration of activity, at different rates. We plan to study this in greater detail.

These data, as a whole, support and confirm the underlying premise of this research program. The proposed mechanism of reversal by pyruvate (Fig. 8) or by succinate (Cohen, Farooqui, & Kesler, 1997) is summarized in Fig. 9. Mitochondrial damage (left side of Figure) follows eqns. 1-3. The repair mechanism depends on the generation of NADH (e.g., PDH activity) followed

by transfer of hydrogens to $NADP^+$, which forms NADPH. This reaction is catalyzed by the enzyme transhydrogenase. In intact mitochondria, it is known that NADPH accumulates to a much greater extent than NADH (e.g., Clark & Nicklas, 1970). The NADPH is subsequently used by GSSG reductase to convert GSSG to GSH. The ratio of GSH to GSSG controls the extent of PrSSG accumulation. As GSSG declines and GSH rises, PrSSG is reduced back to PrSH, enabling enzymatic function, such as that of Complex I. The reaction can be chemical and spontaneous. It is also catalyzed by enzymes, such as glutaredoxin (which utilizes GSH as cofactor), thioredoxin and thioredoxin reductase (NADPH), and by protein-disulfide isomerase.

The formation of NADH and, subsequently, NADPH by succinate requires some comment. The mechanism is shown in Fig 10. Succinate and succinate dehydrogenase do not generate NADH. Instead electrons in Complex II are transferred to coenzyme-Q via FAD. However, in the absence of an electron flux from Complex I (i.e., in the absence of pyruvate). it is well established that electrons from succinate undergo "reversed electron flow". In this reaction sequence, reduced Co-Q (that is, Co-QH_2) will send electrons both downstream (Complex III) and upstream (Complex I). The latter reaction, which is a reversal of normal Complex I activity, results in the reduction of NAD^+ to NADH. Thus, both pyruvate and succinate can reverse the formation of PrSSG.

Conclusions: These data verify that reversal of PrSSG occurs at the inner membrane in concordance with restoration of mitochondrial function. The results confirm a basic premise of the research program and set the stage for more detailed evaluation of mechanisms to either prevent mitochondrial damage, or to restore mitochondrial function, after exposure to neurotoxins or in idiopathic Parkinson's disease.

4. Methods: The described studies were based on the following methods:

a. Mitochondria: Rat brain mitochondria were isolated from Sprague-Dawley rats (250-275g) by a minor modification of the method of Clark and Nicklas (1970). The isolation medium consisted of 5 mM Mops (3-(N-morpholino)propanesulfonic acid), containing 0.225 M mannitol, 0.075 M sucrose, and 1.0 mM EGTA, adjusted to pH 7.4 with KOH. Isolation was carried out in the cold at 15,800 x g for 10 min, followed by 15,000 x g for 30 min in the Ficoll gradient, and a final rinse at 15,800 g for 10 min. The isolated mitochondria were suspended in cold Mops buffer at a concentration of 15-20 mg mitochondria protein/ml and maintained in an ice bath until used. The yield was 3-4 mg mitochondrial protein per rat brain.

b. Incubations: Incubations were conducted by dilution of an aliquot of the mitochondrial preparation to 0.5 mg or 1.0 mg mitochondrial protein/ml in the buffered medium. Incubations were generally carried out at 27°C in a volume of 1 ml in plastic tubes (12-ml) on a water bath with gentle shaking (48 oscillations/min) for 15 min. Each experiment consisted of 12-24 samples (generally 3 samples per group).

c. Mitochondrial electron flow: Electron flow was measured by reduction of MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide). Assays were carried out with a modification (Cohen et al., 1997) of the method described by Berridge and Tan (1993). For direct comparison to respiration (viz., Fig. 5), the respiration buffer was used as the medium. Samples were incubated in buffer containing pyruvate/malate (5 mM each) and MTT (0.42 mg/ml) for 5 min at 27°C and, then, the reaction was quenched by the addition of a lysing buffer consisting of 10% (w/v) sodium dodecylsulfate and 45% (v/v) dimethylformamide, adjusted to pH 4.7 with glacial acetic acid. Absorbance readings were taken in duplicate on a plate reader and reported as the difference in absorbance between 550 nm and 620 nm. Spectrophotometry was preceded by a shaking period of 99 s on the plate reader. Individual samples were expressed as a percent of the mean control value in the experiment.

d. Mitochondrial respiration: Respiration was measured in a miniature 0.6 ml chamber system equipped with a magnetic stirrer and maintained at 27°C (Cohen & Kesler, 1999). Oxygen consumption was assessed with a Biological Oxygen Monitor (Yellow Springs Instrument Co.). Measurements were made sequentially after the addition of pyruvate/malate (5 mM each) (State 4 respiration), followed by 0.4 mM ADP (State 3) and, lastly, 10 μM FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone; State 5). In some experiments, ADP was omitted and State 5 respiration was measured directly. Respiratory activity of the stock mitochondrial preparation, which was held on ice, was well maintained and did not change over the course of 3-4 h.

e. Isolation of inner mitochondrial membrane: The inner mitochondrial membrane (mitoplast membrane) was isolated by the method of Sottocasa et al. (1967). The method uses cold hypotonic lysis (10 mm Tris-phosphate buffer at 0°C) to lyse the outer membrane, followed by contraction of the mitoplasts with cold 2 mM ATP plus 2 mM $MgSO_4$ in 1.8 M sucrose to separate the inner and outer membranes. This procedure produces mitoplast "ghosts" which are devoid of mitoplast protein. We verified the absence of protein from the mitochondrial matrix by protein analysis. Following a rinse and centrifugation, the mitoplast ghost layer is isolated, rinsed, and resuspended in 0.25 M sucrose for subsequent analysis of PrSSG.

f. Protein-glutathione mixed disulfides (PrSSG): PrSSG was measured with a modification of the method of Akerboom and Sies (1981). The liberated GSH was measured on the plate reader with a modification of the enzymatic recycling method of Tietze (1969).

g. Pyruvate dehydrogenase (PDH) activity: PDH activity was measured by the method of Hinman and Blass (1981). The method couples NADH production by PDH to the reduction of a tetrazolium dye in the presence of phenazine methosulfate. The mitochondria were isolated by centrifugation, rinsed once in 20 mM potassium phosphate buffer pH 7.0, containing 1 mM EDTA, and stored frozen in the same buffer (-80°C); dithiothreitol was omitted. Following thawing and centrifugation at 12,000 x g for 20 min, the supernatant fluid was assayed for PDH in 50 mM potassium phosphate buffer (pH 7.8). Readings were taken at ambient temperature on a plate reader (ATTC Model 340, SLT Laboratory Instruments, Hillsborough, NC) at 20 second intervals. PDH rates showed an upward curvature for the first 140 seconds; final assay rates were based on the linear portion of the rate curve between 140 and 300 seconds, and consisted of the mean of the running average for the remaining 80-second rates (5 rates).

The plate reader was calibrated (Cf. Hinman and Blass, 1981) with 37.5 μM INT and excess reducing agent (600 μM dithiothreitol). The mean enzymatic activity in 8 independent experiments was 3.73 ± 0.50 (SEM) nmoles/min/mg protein.

h. Mitochondrial protein: Protein was measured by the method of Lowry et al. (1951) and was used to normalize data for both PrSSG and respiration, which were expressed per mg protein.

i. Data analysis: Data are expressed as the mean ± SEM. Statistical assessment was conducted by ANOVA, followed by the Tukey-Kramer multiple comparison test or, where appropriate, by the 2-tailed Student t-test.

KEY RESEARCH ACCOMPLISHMENTS:

- Verification that H_2O_2 is the toxic product generated by MAO. The corresponding aldehydes and ammonia are eliminated from further consideration as toxins to brain mitochondria.
- The conclusions stated above were based on (a) experiments with added catalase, which is a scavenger of H_2O_2, (b) experiments with azide, which inhibits the trace amounts of endogenous catalase associated with brain mitochondrial preparations, and (c) experiments with glucose oxidase, which generates H_2O_2, but not aldehydes or ammonia.
- Demonstration that inhibition of the pyruvate dehydrogenase complex (PDH) by MAO does contribute to our prior experiments (limited to a 15-min exposure at 27°C). .
- However, on longer exposure to MAO, PDH is substantively inhibited. Therefore, suppression of PDH activity, which, in turn would suppress electron flow to Complex I, may contribute to mitochondrial damage after exposure to neurotoxins or in idiopathic Parkinson's disease.
- Demonstration that the recovery of mitochondrial function (repair of mitochondria) is associated with reduction of PrSSG specifically at the inner mitochondrial membrane.

REPORTABLE OUTCOMES:

1. **Manuscript:** Cohen G & Kesler N (2000) Inhibition of pyruvate dehydrogenase by MAO, J. Free Radical Biology & Medicine (in review).
2. **Manuscript:** Cohen G (2000), Oxidative stress, mitochondrial respiration, and Parkinson's disease, In: Reactive Oxygen Species: From Radiation to Molecular Biology (C.C. Chiueh, Ed.), Ann. New York Acad. Sci. 899: 112-120 (previously reported "in press").
3. **Manuscript:** Cohen G & Kesler N (1999) Monoamine oxidase and mitochondrial respiration. J. Neurochem., 73: 2310-2315 (previously reported "in press").
4. **Manuscript:** Cohen G & Kesler N (1999), Monoamine oxidase (MAO) inhibits mitochondrial respiration, In: Oxidative/Energy Metabolism in Neurodegenerative Disorders (J. Blass, Ed.), Ann. New York Acad. Sci. 893: 273-278 (previously reported "in press").
5. **(Abstract & Poster presentation)** Cohen G, Han S-K & Kesler N (2000) Signaling by hydrogen peroxide via the tissue thiol redox state. (Symp. on Hydrogen Peroxide as an Intracellular Signaling Molecule), Joint meeting Amer. Soc. Biochem. & Molec. Biol. (ASBMB) and Amer. Soc. Pharmacol. & Exptl. Therap. (ASPET), Boston, MA, June 4-8.
6. **(Abstract & Poster presentation)** Cohen G & Kesler N (2000) Inhibition of mitochondrial pyruvate dehydrogenase by MAO, 6th Ann. Mtg. Oxygen Society, San Diego, CA, Nov. 16-20.

CONCLUSIONS

The basic premise of this research is that the turnover of dopamine (DA) by monoamine oxidase (MAO) places an oxidative stress on the mitochondria of DA neurons. The generation of H_2O_2 by MAO leads initially to formation of glutathione disulfide (GSSG) and, subsequently, to protein-glutathione mixed disulfides (PrSSG). Loss of essential protein thiol groups (PrSH), such as those required for proper function of Complex I, compromises mitochondrial electron transport and respiration. In turn, mitochondrial dysfunction contributes to the progression of Parkinson's disease and to the damaging effects of environmental neurotoxins.

The research effort in the 1st year of this USAMRMC grant showed that MAO substrates (1) suppressed both mitochondrial respiration and electron transport, (2) elevated mitochondrial PrSSG, and (3) that MAO inhibitors were protective. Work in 2nd year (1) verified the pivotal role of H_2O_2 in experiments with catalase and azide and (2) showed for the first time that pyruvate dehydrogenase (PDH), as well as complex I, are detrimentally affected by MAO. In addition, and most important, it was shown that (3) reversal of damage during the metabolism of pyruvate or succinate is accompanied by removal (reversal) of PrSSG from the inner mitochondrial membrane. The new laboratory findings support the working hypothesis and help to clarify the pathophysiology of neurodegenerative mechanisms affecting DA neurons. Over the longer range, the new leads concerning (a) the thiol redox state of mitochondria and (b) an understanding of mechanisms that reverse damage, can lead to improved methods to protect DA neurons from environmental neurotoxins and from the ravages of Parkinson's disease.

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FIGURE LEGENDS

Fig. 1. Suppression of MAO-mediated damage by catalase. Dopamine (DA) was used as substrate for MAO. Mitochondria were exposed to 500 μM DA at 27°C for 15 min., both without and with added catalase (10 μg/mL). Pyruvate-supported electron flow was measured in isolated mitochondria with the MTT assay. Statistical assessment was by 2-tailed Student t-test: *$p<0.01$ vs. untreated control; **$p<0.01$ vs. DA alone

Fig. 2. Potentiation of MAO-mediated damage by azide: Time course. Dopamine (DA) was used as substrate for MAO. Mitochondria were exposed to 500 μM DA at 27°C for 20-60 min, both without and with added sodium azide (0.5 mM). Pyruvate-supported electron flow was measured in isolated mitochondria with the MTT assay. Potentiation of MAO-mediated mitochondrial damage (inhibition of electron flow) by azide was significant ($p < 0.01$) at all time points (Tukey-Kramer multiple comparison test).

Fig. 3. Potentiation of MAO-mediated damage by azide: Concentration-response curve. Dopamine (DA) was used as substrate for MAO. Mitochondria were exposed to 25-500 μM DA at 27°C for 15 min, both without and with added sodium azide (0.5 mM). Pyruvate-supported electron flow was measured in isolated mitochondria with the MTT assay. Potentiation of MAO-mediated mitochondrial damage (inhibition of electron flow) by azide was significant ($p < 0.01$) at 100, 200, and 500 μM DA (Tukey-Kramer multiple comparison test).

Fig. 4. Inhibition of mitochondrial electron flow by glucose/glucose oxidase, with potentiation by azide. Glucose (1.1 mM) and glucose oxidase (2.5 μg/mL) were substituted for MAO/dopamine and mitochondria were incubated as indicated for 15 min.
*$p<0.01$ vs. untreated control; **$p<0.01$ vs. glucose/glucose oxidase alone (Tukey-Kramer multiple comparison test).

Fig. 5. Effect of MAO activity on State 5 respiration and lack of effect on pyruvate dehydrogenase (PDH) activity. Tyramine (500 μM) was used as substrate for MAO. Mitochondria were exposed to MAO activity for 15 min at 27°C.
*$p<0.01$ vs. control (n=9/group, 2-tailed t-test with Welch correction). Results for PDH activity did not achieve statistical significance (p=0.071, n=15/group).

Fig. 6. Time course for inhibition of mitochondrial PDH by MAO. Conditions as in Fig. 5, except that the time was extended to 22.5 min and 30 min.
*$p<0.01$ vs. control (n=6/group at 22.5 min and n=9/group at 30 min). As in Fig. 5, results at 15 min did not achieve statistical significance (n=16/group). Statistical analysis was carried out by ANOVA followed by the Bonferroni multiple comparison test, comparing experimental and corresponding control specimens incubated for identical times

Fig. 7. MAO inhibitors (MAOI) block the suppression of mitochondrial PDH activity by tyramine. Mitochondria were exposed to MAO activity with 500 μM tyramine as substrate for 30 min at 27°C. Where indicated (MAOI) a mixture of 2 μM clorgyline and 2 μM pargyline was used to inhibit MAO-A and MAO-B, respectively.

* $p<0.001$ vs. control (n=10-12/group). Statistical assessment was performed by ANOVA followed by Tukey-Kramer multiple comparison test.

Fig. 8. Proposed scheme for the inhibition of mitochondrial electron flow and respiration by MAO, and for reversal of damage by pyruvate or succinate. Suppression of mitochondrial function is based on the formation of protein-glutathione mixed disulfides (PrSSG) with essential thiol groups of Complex I and other proteins. Reversal of damage follows from reduction of glutathione disulfide (GSSG) by GSSG reductase, utilizing NADPH generated during the metabolism of pyruvate or succinate. Succinate generates NADPH by "reverse electron flow", in which reduced coenzyme Q (QH_2), reduces NAD^+ to NADH. Transhydrogenase catalyzes the subsequent reduction of $NADP^+$ to NADPH. Reduction of GSSG to GSH restores a normal thiol redox state and facilitates recovery of mitochondria. NADPH and GSH also serve as cofactors for enzymes that carry out the repair (glutaredoxin, thioredoxin, protein disulfide isomerase).

Fig. 9. Mitochondrial damage by MAO and proposed mechanism of repair by pyruvate or succinate. See text for details.

Fig. 10. Reversed electron flow initiated by succinate causes reduced CoQ to generate NADH from NAD^+. See text for details.

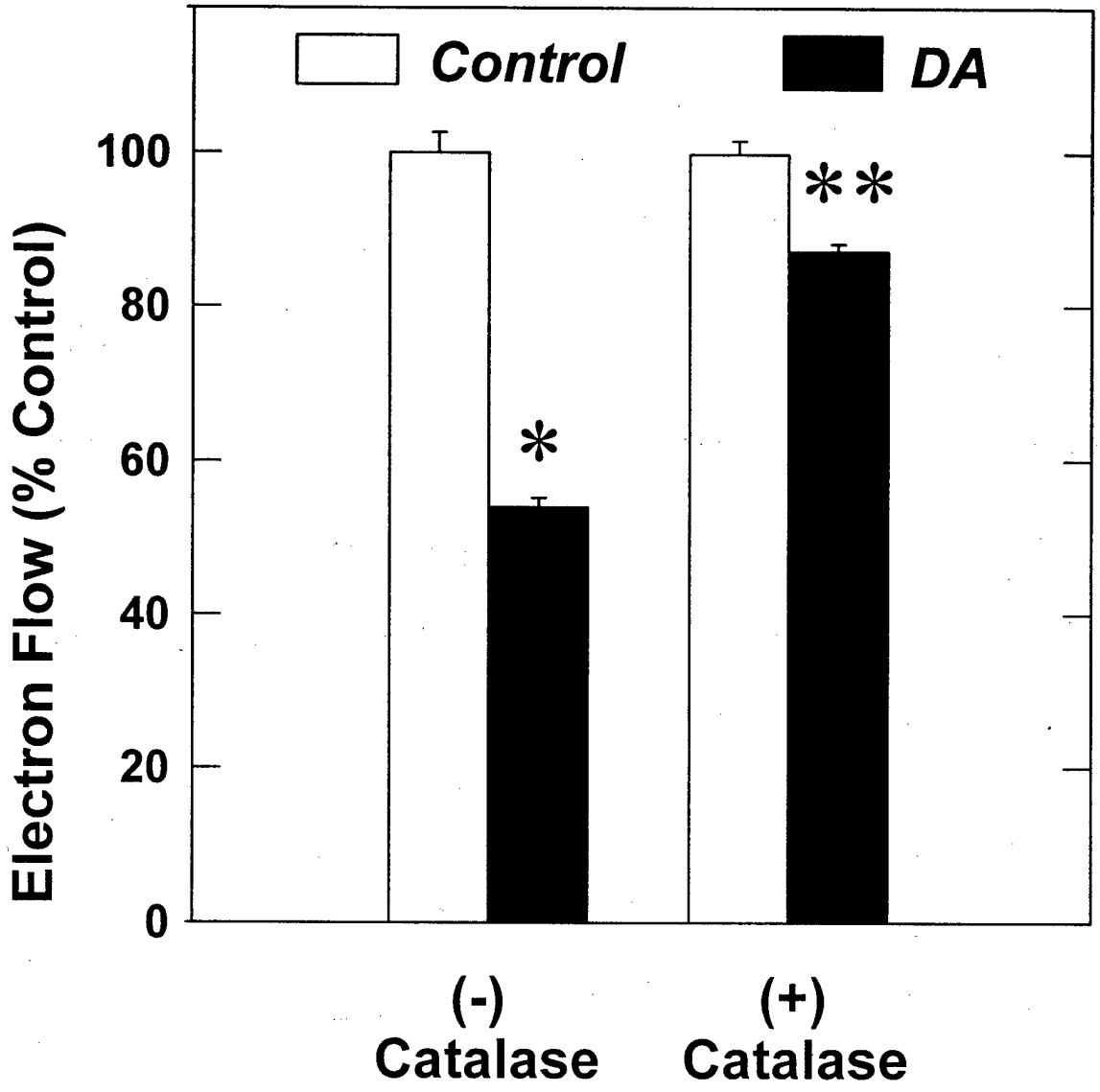


Figure 1. Suppression of MAO-mediated damage by catalase.

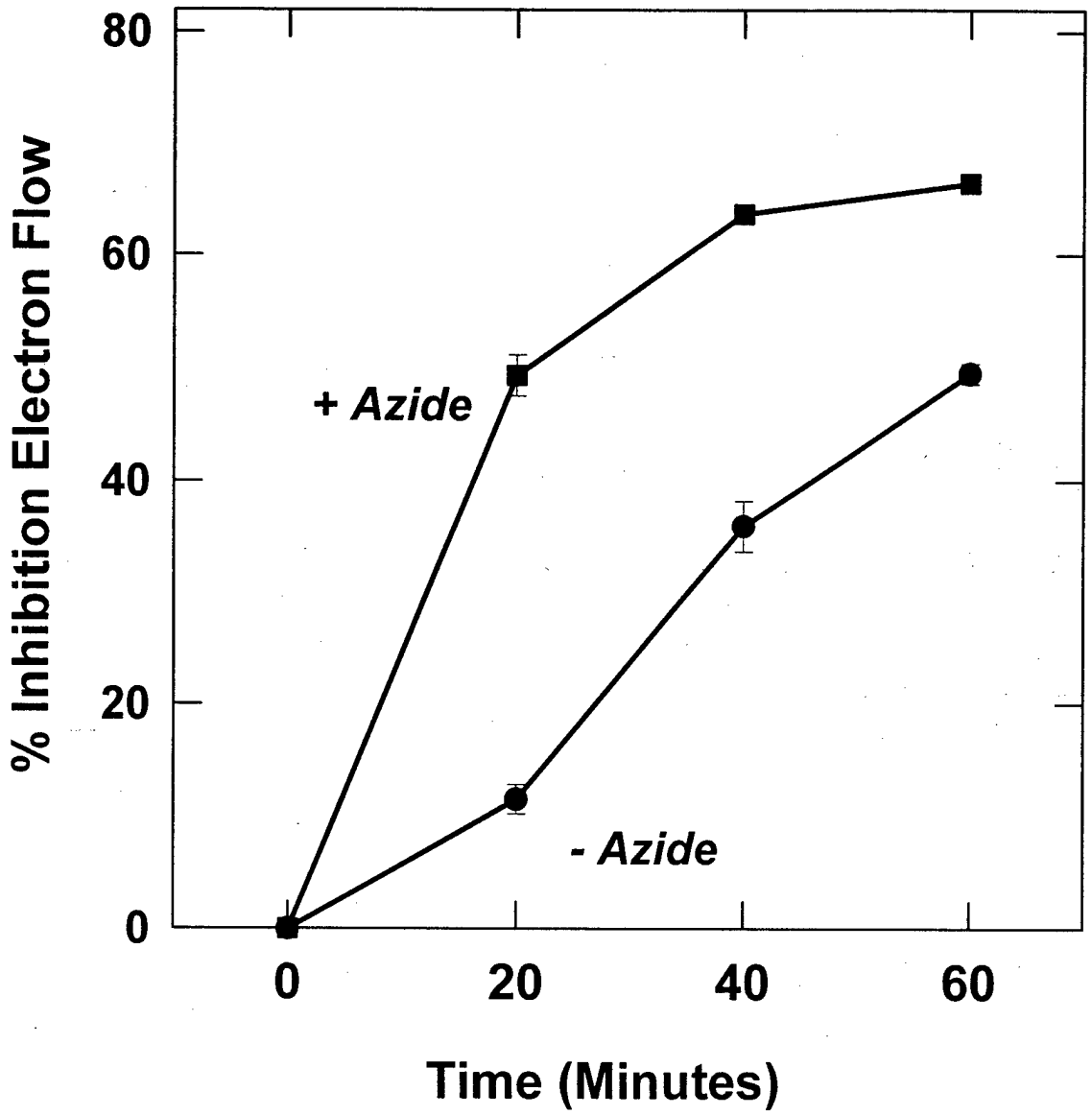


Figure 2. Potentiation of MAO-mediated damage by azide: Time course.

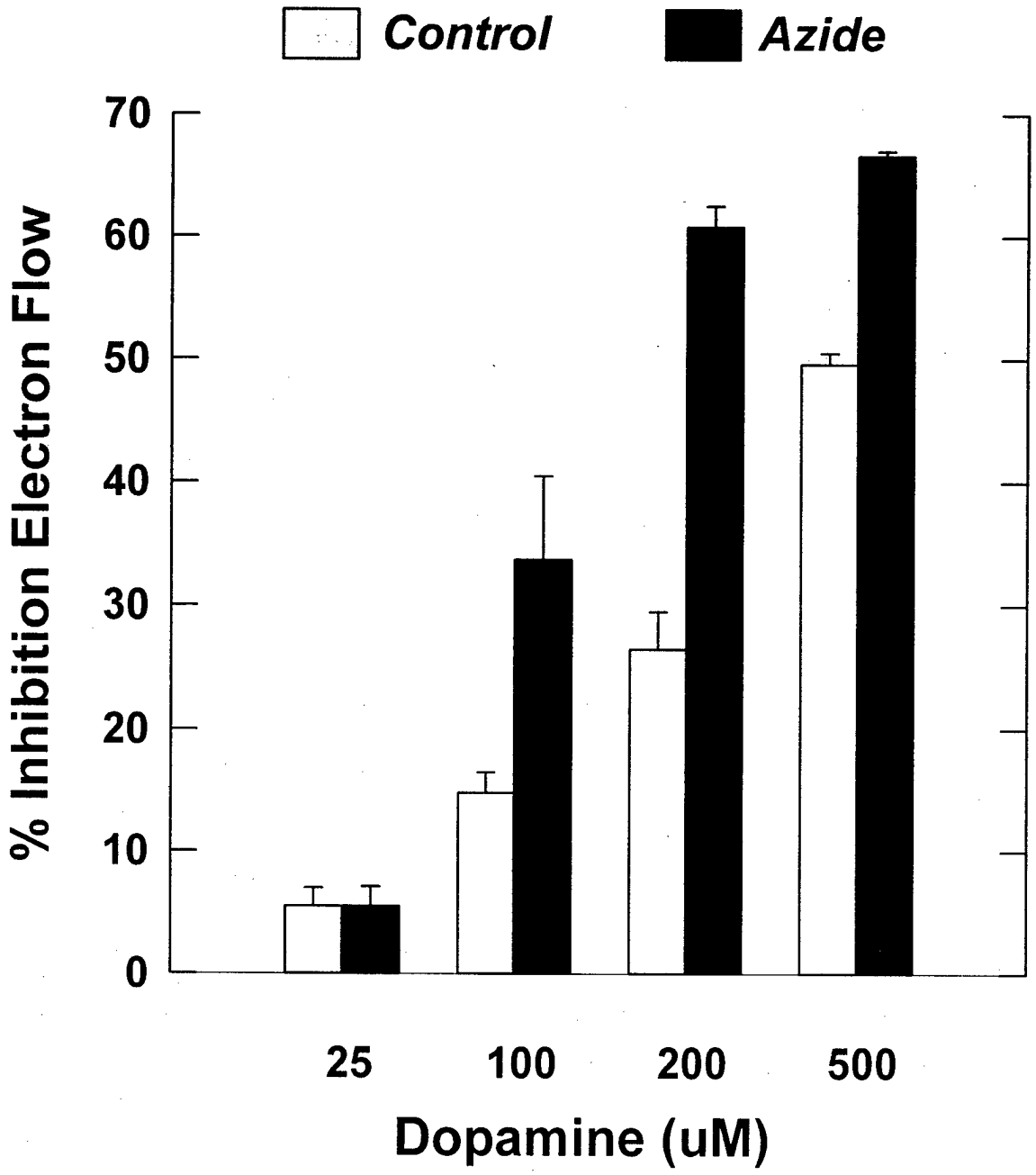


Figure 3. Potentiation of MAO-mediated damage by azide: Concentration-response curve.

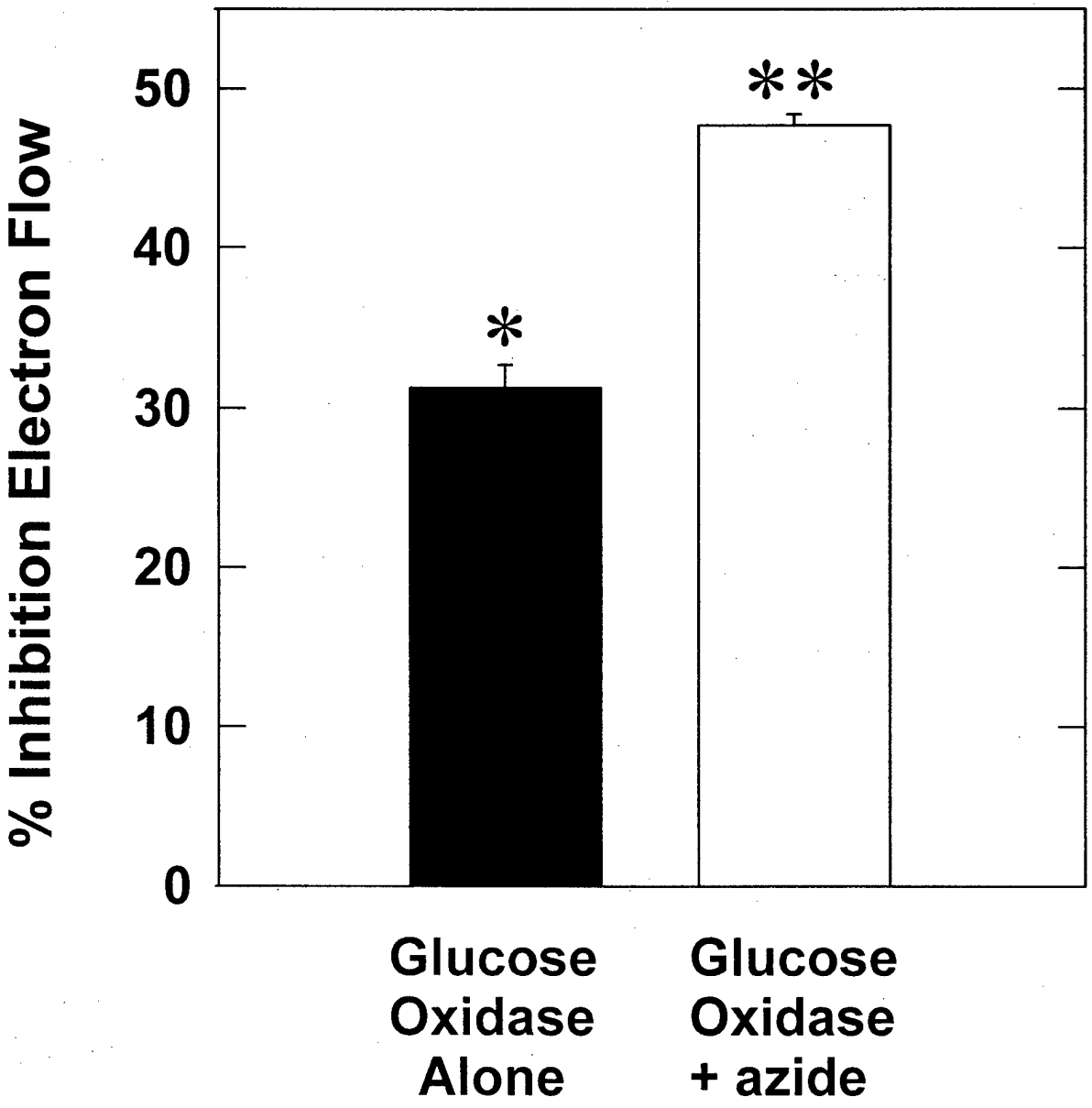


Figure 4. Mitochondrial damage evoked by glucose/glucose oxidase, with potentiation by azide.

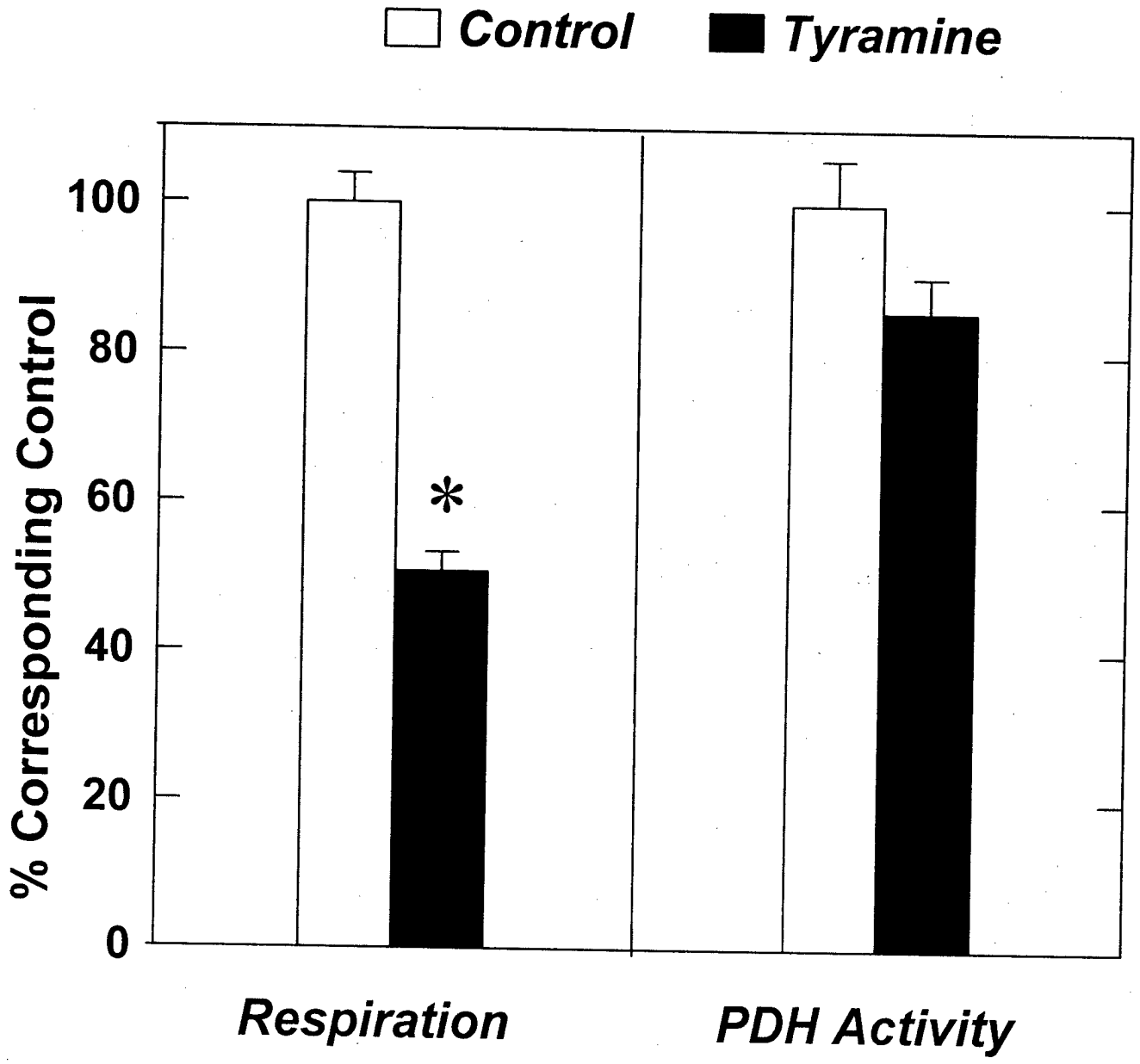


Figure 5. Effect of MAO activity on respiration and lack of effect on pyruvate dehydrogenase activity.

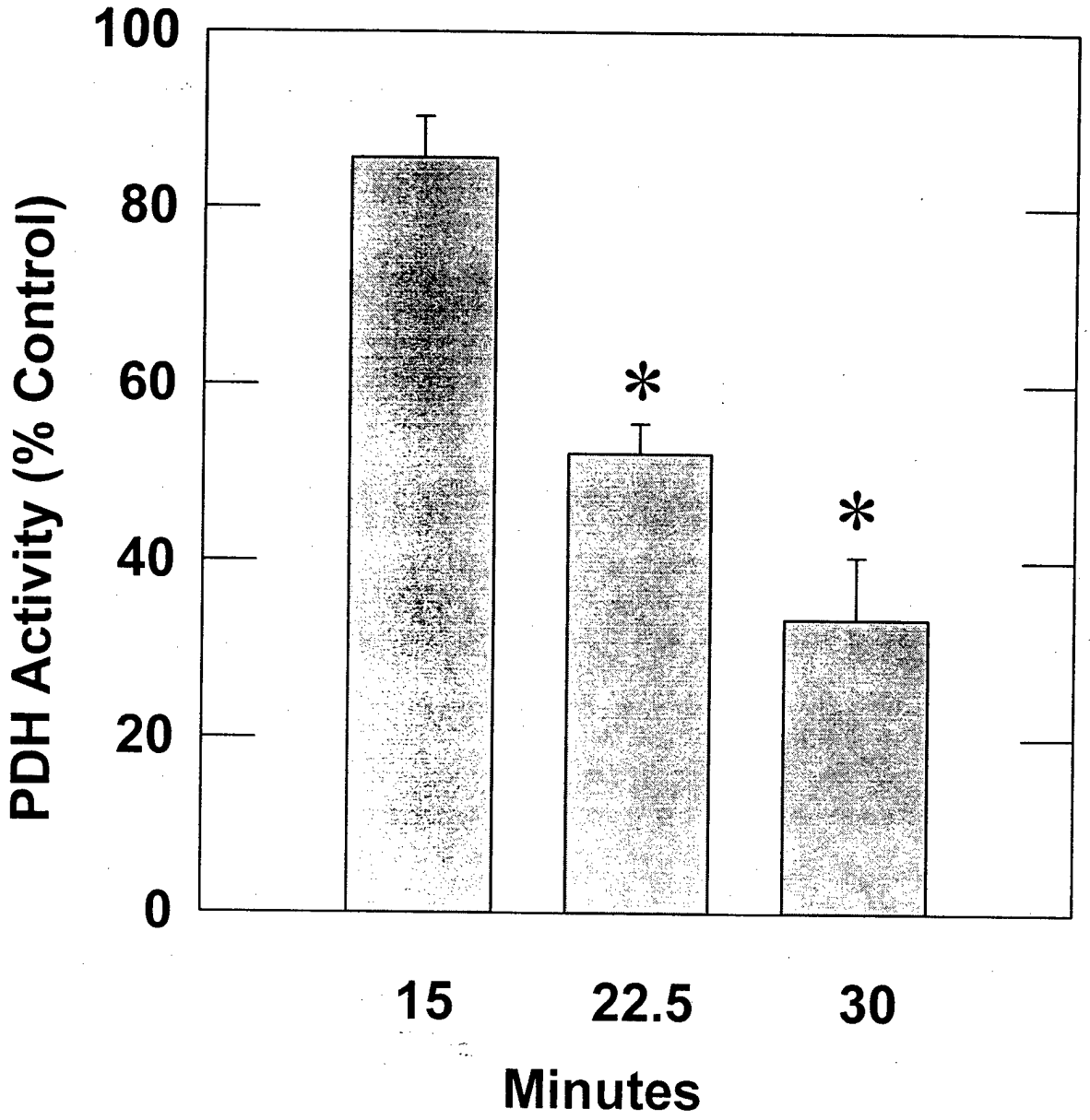


Figure 6. Time course for inhibition of mitochondrial PDH by MAO.

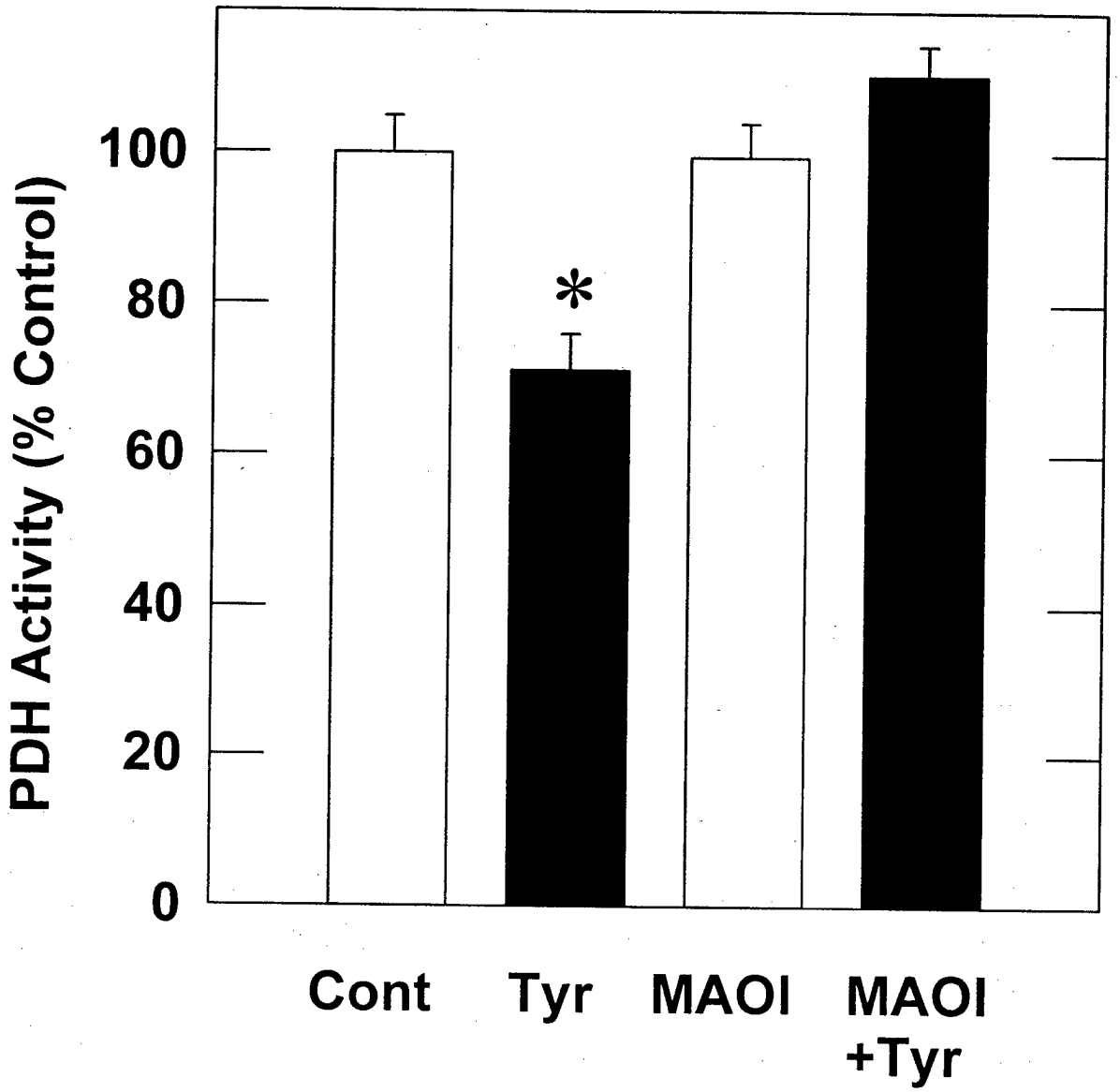
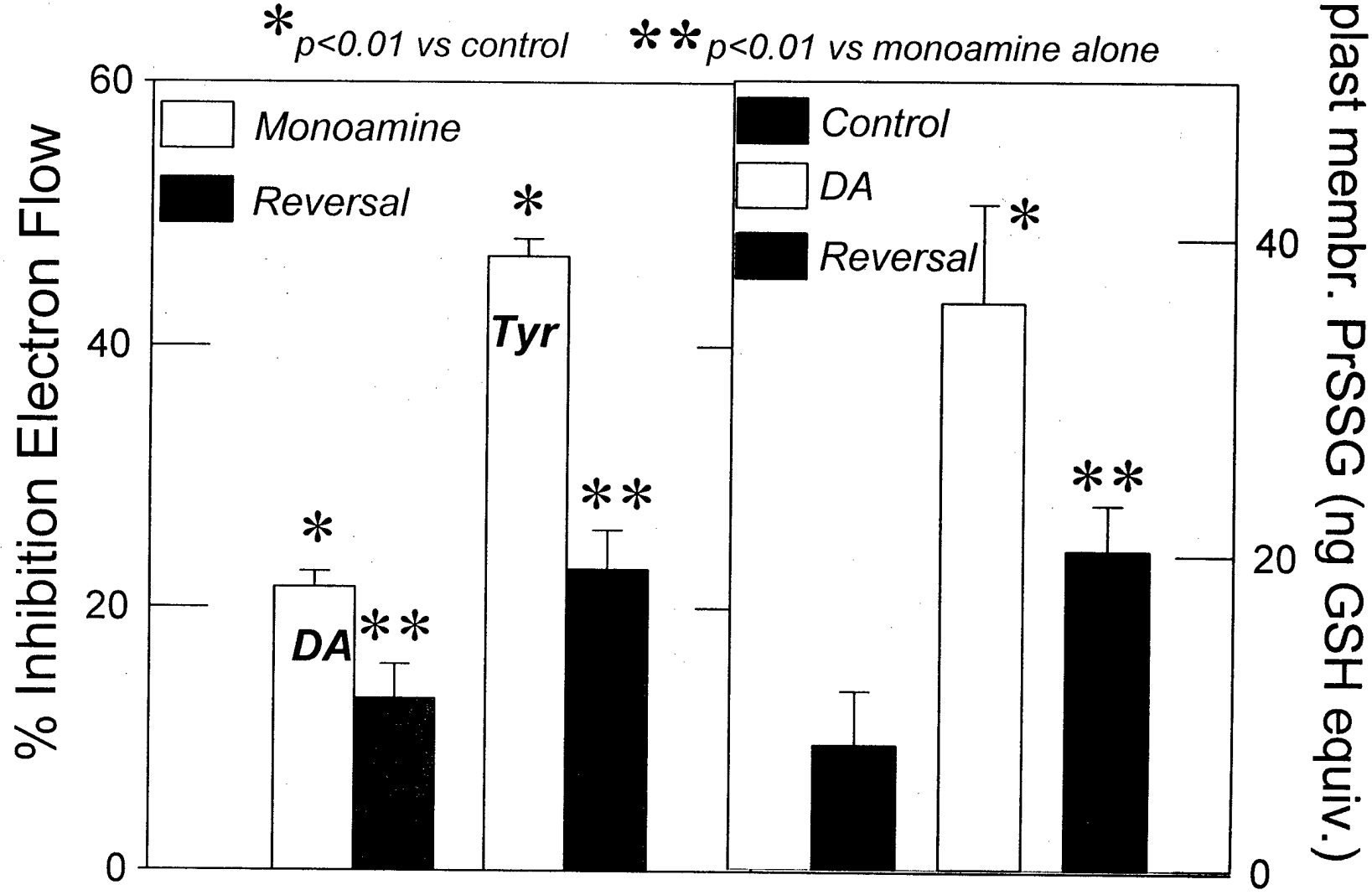


Figure 7. MAO inhibitors (MAOI) protect mitochondrial PDH from inhibition by tyramine.

Figure 8. Reversal of damage by pyrvate/malate (15' @27°C): Electron flow (left) & PrSSG (right, inner membrane).



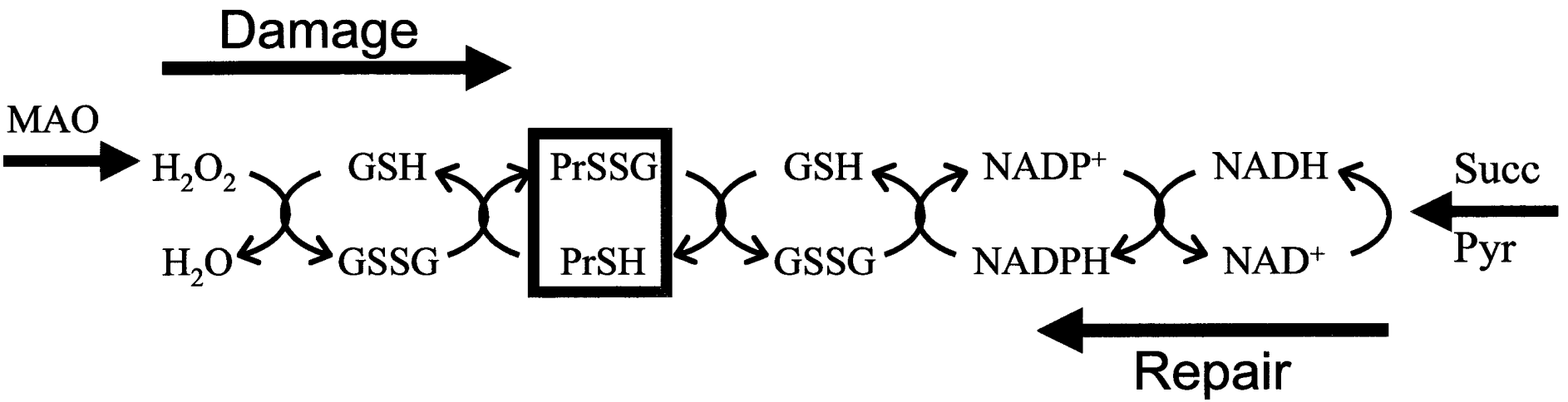


Figure 9. Mitochondrial damage by MAO and proposed mechanism of repair by pyruvate or succinate.

REVERSED ELECTRON FLOW

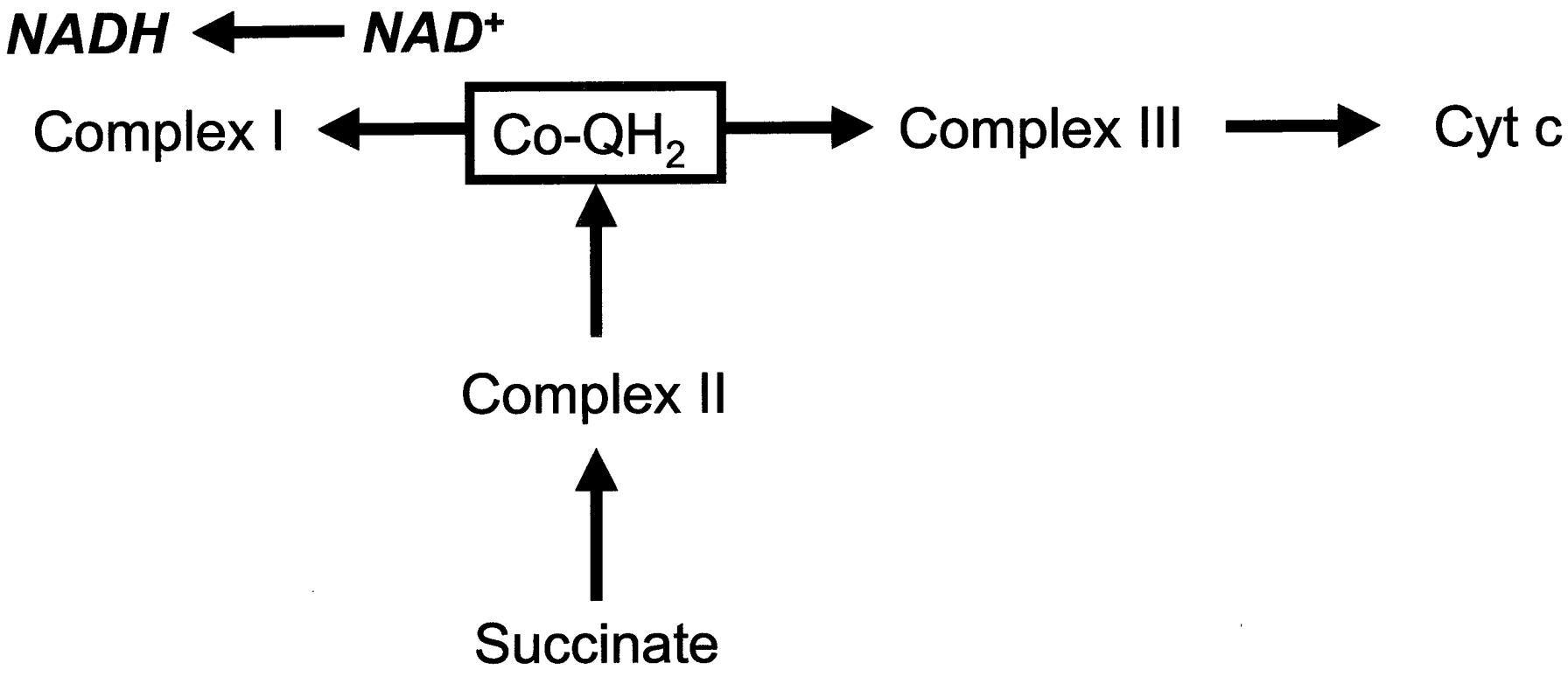


Figure 10. Reversed electron flow initiated by succinate causes reduced CoQ to generate NADH from NAD+

APPENDIX

The appendix consists of 2 abstracts from meeting poster presentations and 3 reprints:

Abstracts:

1. Cohen G, Han S-K & Kesler N (2000) Signaling by hydrogen peroxide via the tissue thiol redox state. (Symp. on Hydrogen Peroxide as an Intracellular Signaling Molecule), Joint meeting Amer. Soc. Biochem. & Molec. Biol. (ASBMB) and Amer. Soc. Pharmacol. & Exptl. Therap. (ASPET), Boston, MA, June 4-8. .
2. Cohen G & Kesler N (2000) Inhibition of mitochondrial pyruvate dehydrogenase by MAO, 6th Ann. Mtg. Oxygen Society, San Diego, CA, Nov. 16-20.

Manuscripts:

1. Cohen G & Kesler N (1999) Monoamine oxidase and mitochondrial respiration. J. Neurochem., 73: 2310-2315 (previously reported "in press").
2. Cohen G & Kesler N (1999), Monoamine oxidase (MAO) inhibits mitochondrial respiration, In: Oxidative/Energy Metabolism in Neurodegenerative Disorders (J. Blass, Ed.), Ann. New York Acad. Sci. 893: 273-278 (previously reported "in press")
3. Cohen G (2000), Oxidative stress, mitochondrial respiration, and Parkinson's disease, In: Reactive Oxygen Species: From Radiation to Molecular Biology (C.C. Chiueh, Ed.), Ann. New York Acad. Sci. 899: 112-120 (previously reported "in press")

Appendix: Abstract (1)

Joint Meeting
American Society Biochemistry & Molecular Biology (ASBMB)
American Society Pharmacology & Experimental Therapeutics (ASPET) Boston, MA June 4-8, 2000

Symposium on Hydrogen Peroxide as an Intracellular Signaling Molecule

SIGNALING BY HYDROGEN PEROXIDE VIA THE TISSUE SULFHYDRYL REDOX STATE

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Department of Neurology and Center for Neurobiology,
Mount Sinai School of Medicine, New York, NY 10029

H_2O_2 can act as a signaling molecule via reversible changes between thiols and disulfides. Typically, H_2O_2 is detoxified by GSH peroxidase, forming GSSG. GSSG reacts with proteins to generate mixed disulfides (PrSSG) and protein disulfides (PrSSPr). Subsequent enzymatic reduction of -SS- bonds provides a flexible and reversible signaling mechanism. A prime example in E. coli is the OxyR transcription factor, activated by oxidation to an intramolecular disulfide either directly by H_2O_2 or via the tissue thiol redox state (Aslund et al., PNAS 1999).

The following experiments illustrate an antioxidant action of H_2O_2 generated by autoxidation: Addition of dopamine or L-Dopa to cell cultures of rat brain evokes a rise in GSSG and PrSSG. Although one would expect GSH to fall, to the contrary, GSH levels rise (30-100%), mediated by downstream signaling via protein kinase C. The ability to up-regulate GSH is shared by compounds that autoxidize. Elevated GSH protects cells against loss in viability when challenged with t-butylhydroperoxide. Thus, a mild oxidative stress evokes a beneficial effect.

H_2O_2 is also well recognized for its toxic potential. In experiments with rat brain mitochondria, oxidation of amines by the outer membrane H_2O_2-generating enzyme, monoamine oxidase (MAO), inhibits state 3 & state 5 respiration, accompanied by a rise in PrSSG. Glucose oxidase can substitute for MAO. These results illustrate the beneficial signaling properties and the contrary toxic properties of H_2O_2. *[Supported by grants from USAMRMC & USPHS].*

Appendix: Abstract (2)

6th Annual Meeting of the The Oxygen Society:
San Diego, CA, Nov. 16-20, 2000

INHIBITION OF MITOCHONDRIAL PYRUVATE DEHYDROGENASE BY MONOAMINE OXIDASE

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Monoamine oxidase (MAO) is localized to the outer mitochondrial membrane and is responsible for the metabolism of neurotransmitters, such as dopamine (DA), in the central nervous system. Because MAO generates H2O2, it serves as a source of an oxidant stress. Experiments with brain mitochondria established that MAO elevates protein-glutathione mixed disulfides and suppresses pyruvate-dependent respiration (J. Neurochem. 1999). Diminished respiration may be mediated by damage to critical protein thiol groups in either pyruvate dehydrogenase (PDH) or Complex I (NADH-CoQ reductase). The current experiments show that PDH is progressively inhibited and MAO inhibitors are protective, but damage is delayed in time compared to respiration. After 15 min exposure to 500 μM tyramine (a mixed MAO-A/MAO-B substrate), respiratory inhibition is 3.4-fold greater than suppression of PDH. Moreover, the small inhibition of PDH does not achieve statistical significance. Damage to PDH is more pronounced and significant after longer exposure times (e.g., 30 min). Therefore, early suppression of respiration is best attributed to damage downstream at Complex I. We conclude that although PDH and Complex I are both targets for MAO-mediated damage, Complex I is more susceptible. In Parkinson's disease, surviving DA neurons exhibit increased turnover of DA associated with increased metabolism by MAO. Therefore, the current observations may reflect on the known brain defect in Complex I in Parkinson's disease and on the origin of the mitochondrial lesion. *[Supported by a grant from the USAMRMC].*

Journal of Neurochemistry
Lippincott Williams & Wilkins, Inc., Philadelphia

Monoamine Oxidase and Mitochondrial Respiration

Gerald Cohen and Natasa Kesler

Department of Neurology and Center for Neurobiology, Mount Sinai School of Medicine, New York, New York, U.S.A.

Abstract: Mitochondrial defects encompassing complexes I–IV of the electron transport chain characterize a relatively large number of neurodegenerative diseases. The relationships between mitochondrial lesions and recently described genetic alterations have not yet been defined. We describe a general mechanism whereby the enzymatic metabolism of neurotransmitters by monoamine oxidase (MAO) damages mitochondria, altering their protein thiol status and suppressing respiration. In these experiments, incubation of rat brain mitochondria with tyramine (a mixed MAO-A/MAO-B substrate) for 15 min at 27°C suppressed state 3 respiration by 32.8% and state 5 respiration by 40.1%. These changes were accompanied by a 10-fold rise in protein–glutathione mixed disulfides. Direct comparison of effects on respiration and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction during electron flow gave similar results. It is suggested that certain mitochondrial lesions may derive from the natural turnover of monoamine neurotransmitters in susceptible individuals. **Key Words:** Mitochondria—Respiration—Monoamine oxidase—Tyramine—Dopamine—Glutathione—Protein mixed disulfides—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).
J. Neurochem. **73,** 2310–2315 (1999).

Mitochondrial defects associated with complexes I–IV of the respiratory chain occur in a number of neurodegenerative diseases including Parkinson's disease, Huntington's disease, Friedreich's ataxia, and hereditary spastic paraplegia (Arenas et al., 1998; Schapira, 1998, 1999). Evidence for mitochondrial involvement also exists for Alzheimer's disease and amyotrophic lateral sclerosis (Cassarino and Bennett, 1999). Moreover, several animal models of neurodegenerative disease are based on mitochondrial toxins such as MPTP, which inhibits complex I of the electron transport chain, producing an animal model for Parkinson's disease, or 3-nitroproprionic acid and malonate, which inhibit complex II, producing models for Huntington's disease (Schulz et al., 1997). Therefore, mitochondrial defects appear to play primary roles in disease expression and progression. Defects in cellular respiration lead to diminished ATP production, increased sensitivity to oxidative stress, and, eventually, apoptotic or necrotic neuronal cell death (Zamzami et al., 1997).

Mitochondrial respiratory defects can be directly inherited or may be acquired as the result of exposure to stressors. We identify a mitochondrial enzyme, monoamine oxidase (MAO), and the turnover of monoamine neurotransmitters by MAO as a source of oxidative stress that can suppress mitochondrial respiration. MAO is a flavoenzyme, localized to the outer mitochondrial membrane (Schnaitman et al., 1967; Ragan et al., 1987). It plays an essential role in the turnover of monoamine neurotransmitters such as dopamine, serotonin, and norepinephrine (Cooper et al., 1996). Oxidative deamination of monoamines by MAO is accompanied by the reduction of molecular oxygen to hydrogen peroxide (Sinet et al., 1980; Hauptmann et al., 1996), a potentially toxic agent.

H_2O_2 is also formed naturally during mitochondrial respiration. Although consumed oxygen is converted to water, a small fraction is diverted to superoxide (Forman and Boveris, 1982), which dismutes, forming H_2O_2. It is estimated that 1–3% of consumed oxygen is converted to H_2O_2 (Chance et al., 1979). H_2O_2 that "leaks" from the electron transport chain can damage mitochondrial proteins and mitochondrial DNA (Sohal et al., 1995; Giulivi and Cadenas, 1998). It is widely believed that the H_2O_2 generated during respiration is responsible for mitochondrial damage in aging, reperfusion injury, and certain disease states (Ku et al., 1993; Richter et al., 1995). Therefore, other cellular sites of H_2O_2 production that may affect mitochondrial function need to be considered. The mitochondrial localization of MAO makes this enzyme uniquely situated to evoke selective mitochondrial damage.

The quantity of H_2O_2 generated by mitochondrial MAO exceeds by a wide margin the amount generated during electron flow. Hauptmann et al. (1996) studied the oxidation of 2 m*M* tyramine by rat brain mitochondria and reported that H_2O_2 production was 48-fold greater than that from succinate during electron transport in the presence of antimycin A. Because antimycin A effectively doubles the rate of H_2O_2 production during electron transport (Giulivi and Cadenas, 1998), the differential in the absence of antimycin would be double or in the

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Abbreviations used: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; MAO, monoamine oxidase; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PrSSG, protein–glutathione mixed disulfides.

range of 100-fold. Formation of the highly reactive hydroxyl radical and damage to mitochondrial DNA have been observed (Hauptmann et al., 1996). Hence, MAO possesses a considerable toxic potential.

In our experiments, tyramine served as substrate for both isoforms of MAO (MAO-A and MAO-B). We observed diminished mitochondrial respiration accompanied by changes in the mitochondrial sulfhydryl status, namely, accumulation of protein–glutathione mixed disulfides (PrSSG). These effects were blocked by inhibition of MAO. These observations imply that monoamine turnover by neurons (and/or glia) can initiate or contribute to mitochondrial defects. In Parkinson's disease, in particular, where monoamine turnover is accelerated (Hornykiewicz and Kish, 1986), MAO may enhance the known defect in mitochondrial complex I. It is important to identify neuronal sources of oxidant stress such as MAO and mechanisms responsible for impairment of mitochondrial function, as they can help to identify deficiencies in protective or repair mechanisms that may underlie mitochondrially based neurodegeneration in susceptible individuals.

MATERIALS AND METHODS

Reagents

HEPES, EGTA, ADP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-(*N*-morpholino)propanesulfonic acid (MOPS), carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), bovine serum albumin fraction V, sodium dodecyl sulfate, and dopamine-HCl were from Sigma (St. Louis, MO, U.S.A.); tyramine HCl was from RBI (Natick, MA, U.S.A.). FCCP was dissolved in 50% (vol/vol) ethanol. The stock solution of pyruvate plus malate was titrated to neutrality with KOH.

Isolation of mitochondria

Mitochondria were isolated from the pooled whole brain (less the cerebellum) of groups of three Sprague–Dawley rats (250–275 g; Taconic Farms, Germantown, NY, U.S.A.) by a minor modification of the method of Clark and Nicklas (1970). The isolation medium consisted of 5 m*M* MOPS, containing 0.225 *M* mannitol, 0.075 *M* sucrose, and 1.0 m*M* EGTA, adjusted to pH 7.4 with KOH. Isolation was carried out in the cold with a refrigerated Sorvall RC24 centrifuge equipped with an SS 34 rotor at 15,800 *g* for 10 min, followed by 15,000 *g* for 30 min in the Ficoll gradient and a final rinse at 15,800 *g* for 10 min. The isolated mitochondria were suspended in cold MOPS buffer at a concentration of 15–20 mg of mitochondrial protein/ml and maintained in an ice bath until used. The yield was 3–4 mg of mitochondrial protein/rat brain.

Incubation

Incubations were conducted by dilution of an aliquot of the mitochondrial preparation to 0.5 or 1.0 mg of mitochondrial protein/ml in the respiration buffer (pH 7.2), which consisted of 5 m*M* HEPES, 125 m*M* sucrose, 50 m*M* KCl, 2 m*M* KH_2PO_4, and 1 m*M* $MgCl_2$ (Moreadith and Fiskum, 1984) with 0.5 mg of bovine serum albumin/ml at 27°C. Incubations were carried out at 27°C in a volume of 1 ml in plastic tubes (12 ml) on a water bath with gentle shaking (48 oscillations/min) for 15 min. Samples were processed individually with immediate assessment of respiration and rotation among the experimental groups. Samples not incubated with MAO inhibitors (2 μM clorgyline plus 2 μM pargyline) received additions of the inhibitors after the incubation was complete, just prior to the measurement of respiration or electron flow. Each experiment consisted of 10–12 samples (3 or 4 samples/group).

Mitochondrial respiration

Respiration was measured in a miniature chamber system (0.6-ml capacity; Instech Labs., Plymouth Meeting, PA, U.S.A.) equipped with a magnetic stirrer and maintained at 27°C. Oxygen consumption was assessed with a YSI model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Measurements were made sequentially after the addition of pyruvate/malate (5 m*M* each) (state 4 respiration) followed by 0.4 m*M* ADP (state 3) and last 10 μM FCCP (state 5). In some experiments, ADP was omitted and state 5 respiration was measured directly. Respiratory activity of the stock mitochondrial preparation, which was held on ice, was well maintained and did not change over the course of 3–4 h.

Mitochondrial electron flow

MTT assays were carried out with a modification (Cohen et al., 1997) of the method described by Berridge and Tan (1993), except that the respiration buffer was used as the medium. Samples were incubated in buffer containing pyruvate/malate (5 m*M* each) and MTT (0.42 mg/ml) for 5 min at 27°C, and then the reaction was quenched by the addition of a lysing buffer (Berridge and Tan, 1993) consisting of 10% (wt/vol) sodium dodecyl sulfate and 45% (vol/vol) dimethylformamide, adjusted to pH 4.7 with glacial acetic acid. Absorbance readings were taken in duplicate on a plate reader (ATTC model 340; SLT Laboratory Instruments, Hillsborough, NC, U.S.A.) and reported as the difference in absorbance between 550 and 620 nm. Spectrophotometry was preceded by a shaking period of 99 s on the plate reader. Individual samples were expressed as a percent of the mean control value in the experiment.

Assays for PrSSG and for protein

PrSSG was measured with a modification of the method of Akerboom and Sies (1981). The liberated GSH was measured on the plate reader with a modification of the enzymatic recycling method of Tietze (1969). Protein was measured by the method of Lowry et al. (1951) and was used to normalize data for both PrSSG and respiration, which were expressed per milligram of protein.

Data evaluation

Data are expressed as means ± SEM. Statistical assessment was conducted by the Tukey–Kramer multiple comparison test or, where appropriate, by the two-tailed Student's *t* test.

RESULTS

Effect of MAO activity on mitochondrial respiration

Mitochondrial respiration is normally divided into stages or states. In our studies, we refer to state 4 (addition of substrate alone), state 3 (substrate + ADP), and state 5 (uncoupled or maximally stimulated respiration). The respiratory control ratios (state 3/state 4) of freshly isolated mitochondria were in the range of 5.5–7.0 with 5 m*M* pyruvate plus 5 m*M* malate as substrate. State 3 respiration was in the range of 72–103 ng-atoms of oxygen/min/mg of protein. Figure 1 shows results from experiments in which mitochondria were incubated with 500 μM tyramine, with

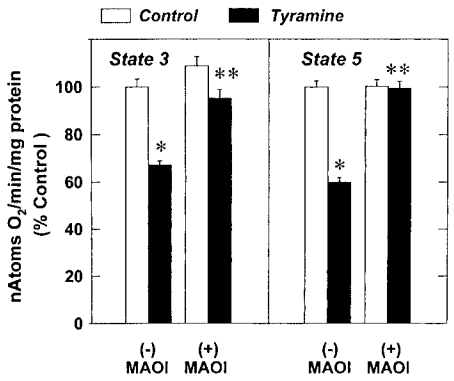

FIG. 1. Mitochondrial respiration with pyruvate/malate (5 m*M* each) as substrate after incubation of samples either without (control) or with 500 μM tyramine for 15 min at 27°C. Where indicated, MAO inhibitors (MAOI) were present (2 μM clorgyline plus 2 μM pargyline) during the incubation procedure. MAO inhibitors were added to all other samples just prior to the measurement of mitochondrial respiration. For state 3 respiration, 0.4 m*M* ADP was added (n = 6; two independent experiments); for state 5, 10 μM FCCP was added (n = 11; four independent experiments). State 5 respiration was assessed in the presence of ADP in two experiments and in its absence in two experiments. Statistical assessment was by ANOVA followed by the Tukey–Kramer multiple comparison test. *$p < 0.001$ vs. untreated control; **$p < 0.001$ vs. corresponding tyramine-treated samples without MAO inhibitors.

and without MAO inhibitors, at 27°C for 15 min; 500 μM was chosen because this is the estimated level of monoamine neurotransmitter in the cytosol of catecholamine neurons (Cohen et al., 1997). State 3 and state 5 respiration were measured with pyruvate/malate (5 m*M* each) as substrate. Tyramine is a mixed MAO-A/MAO-B substrate (Tipton et al., 1976); therefore, a mixture of clorgyline (selective MAO-A inhibitor) and pargyline (selective MAO-B inhibitor) was used to inhibit MAO.

State 3 respiration was suppressed by 32.8 ± 1.7% (mean ± SEM, n = 6) and state 5 respiration by 40.1 ± 1.9% (n = 11) ($p < 0.001$) compared with the corresponding control samples without tyramine (Fig. 1). Inclusion of the MAO inhibitors clorgyline and pargyline (2 μM each) fully protected the mitochondria ($p < 0.001$). MAO inhibitors by themselves did not affect mitochondrial respiration (not shown). Representative oxygen electrode tracings are presented in Fig. 2. Normally, mitochondrial respiration and synthesis of ATP are coupled on demand to the presence of ADP. Figure 2 illustrates state 3 respiration upon the addition of ADP and state 5 respiration after uncoupling with FCCP. Figure 2 also demonstrates the diminution in both state 3 and state 5 respiration after exposure to tyramine.

Isolated mitochondria are delicate and susceptible to loss in respiratory function during incubation procedures. Therefore, samples were evaluated for the change in respiration due to the incubation conditions alone (comparison of incubated versus nonincubated controls). The experiments with tyramine were limited to 15 min to limit this form of mitochondrial damage. Incubation for 15 min at 27°C decreased respiration by 27.3 ± 0.7% for state 3 and 21.7 ± 1.5% for state 5 ($p < 0.01$, n = 5/group, two experiments). However, the data in Fig. 1 are expressed as the effects of tyramine relative to incubated controls; therefore, changes due to experimental conditions, unrelated to tyramine, cancel out. Nonetheless, the loss of a highly vulnerable fraction of respiratory activity may cause the effect of tyramine to be underestimated in Fig. 1. The stock concentrated suspension of mitochondria (15–20 mg of protein/ml) in MOPS buffer, held on ice, was stable and did not lose respiratory activity over the course of the experiments (3–4 h).

Comparison of mitochondrial electron flow and respiration

A prior report described the effect of incubation with tyramine, dopamine, or benzylamine on the ability of mitochondria to reduce MTT, a formazan dye, during electron flow (Cohen et al., 1997). Inhibition was observed when electron flow was initiated either at complex I (pyruvate) or at complex II (succinate); however, complex I showed a greater susceptibility to damage. Figure 3 presents results of experiments in which respiration and electron flow were directly compared. Tyramine had a similar effect on both respiration and MTT reduction: Inhibition of respiration by 500 μM tyramine after 15 min was 24.6 ± 1.2%, whereas inhibition of electron flow was 24.7 ± 2.4%.

The respiratory data in Fig. 3 were obtained in the presence of FCCP as an uncoupling agent. FCCP was also present in samples analyzed with MTT to make direct comparison between the two assays under comparable conditions. It is known that FCCP partially suppresses reduction of MTT by rat brain mitochondria (Liu et al., 1997). In our experiments, FCCP diminished MTT reduction by 39.2 ± 3.4% ($p < 0.01$, n = 6). However, direct comparison of experimental results with and without FCCP added after a 15-min exposure to tyramine indicated that inhibition of electron transport was 24.7 ± 3.0% in the presence of FCCP and 29.4 ± 1.6% in its absence (n = 6/group). Therefore, the basic phenomenon was essentially the same whether or not FCCP was present. In additional experiments, results were similar when FCCP was added either prior to or after incubation

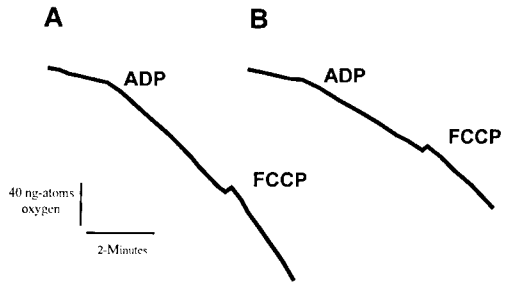

FIG. 2. Representative oxygen electrode tracings in the presence of pyruvate plus malate after incubation at 27°C for 15 min, both without (**A;** control) and with (**B**) 500 μM tyramine. Where indicated, ADP (0.4 m*M*) and FCCP (10 μM) were added.

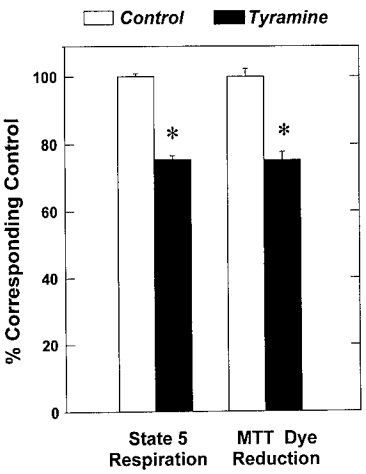

FIG. 3. Direct comparison of mitochondrial state 5 respiration and electron transport (MTT assay) after incubation of samples with and without tyramine (500 μM) for 15 min at 27°C (n = 6; two experiments). The mean difference (550 nm − 620 nm) for control samples in the MTT assay was 0.083 absorbance unit. *$p < 0.001$, two-tailed Student's t test.

with tyramine. Therefore, FCCP does not interfere with the assessment of damage to electron transport.

Changes in protein thiol redox status

Glutathione peroxidase functions in intact mitochondria to remove H_2O_2. GSSG, formed as the result of detoxification of MAO-generated H_2O_2, can react with protein thiols to form PrSSG (Reed, 1990). Disulfide linkages with protein would be expected to suppress thiol-dependent enzymatic activity such as that exhibited by complex I or pyruvate dehydrogenase (Gutman et al., 1970; Ali et al., 1993). We studied the formation of PrSSG in mitochondria. The results of Fig. 4 show that PrSSG levels increased rapidly and progressively during

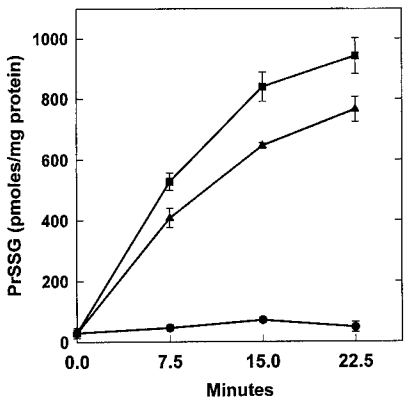

FIG. 4. Formation of PrSSG in mitochondria during incubation at 27°C with 500 μM tyramine (■) or 500 μM dopamine (▲) compared with control samples (●). Results are pooled from two experiments (n = 4/group). Basal level of PrSSG at zero time was 29.8 ± 17.0 pmol/mg of protein. The elevation in PrSSG in samples incubated with tyramine or dopamine compared with corresponding controls was significant at all time points ($p < 0.001$).

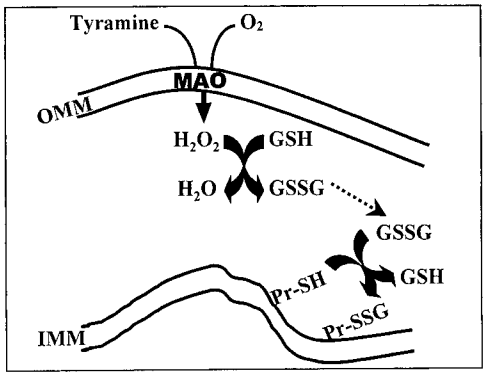

FIG. 5. Proposed scheme for the inhibition of mitochondrial respiration by MAO and tyramine. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane.

incubation of mitochondria with either tyramine or dopamine. The increase over corresponding controls after 15 min (the time at which respiration was measured) was >10-fold. This observation is commensurate with secondary oxidative damage based on a change in the thiol status of proteins. MAO inhibitors completely suppressed PrSSG formation (not shown). Figure 5 is a schematic representation of the proposed mechanism for formation of PrSSG and inhibition of mitochondrial respiration. After oxidation of GSH at the outer mitochondrial membrane, GSSG can diffuse to the inner membrane and form PrSSG. The latter reaction is catalyzed by thioredoxins and protein disulfide isomerases (Holmgren, 1985; Rabenstein and Millis, 1995).

DISCUSSION

The presence of both GSH and GSH peroxidase in mitochondria is well established (Flohe and Schlegel, 1971; Panfili et al., 1991); mitochondria contain up to 26% of the total tissue enzyme (Flohe and Schlegel, 1971). H_2O_2 generated by mitochondria is normally removed by GSH peroxidase (Maker et al., 1981; Sandri et al., 1990; Hauptmann et al., 1996), resulting in the formation of GSSG (Eq. 1). When MAO substrates are added to rat brain mitochondria, H_2O_2 appears in the medium and GSH levels fall (Sandri et al., 1990); these effects are blocked by inhibition of MAO. Accumulation of GSSG was observed in isolated rat liver or brain mitochondria incubated with MAO substrates (Werner and Cohen, 1991, 1993); inhibition of MAO prevented the rise in GSSG. Formation of GSSG was also observed after incubation of mitochondria with *tert*-butylhydroperoxide (Olafsdottir and Reed, 1988), which served as an organic peroxide substrate for glutathione peroxidase. GSSG is normally retained within mitochondria (Olafsdottir and Reed, 1988) and reacts therein with protein thiols (Reed, 1990) to form PrSSG (Eq. 2). Figure 4 illustrates the sharp rise in PrSSG evoked by MAO with either tyramine or dopamine as substrate.

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \qquad (1)$$

$$GSSG + PrSH \rightarrow PrSSG + GSH \quad (2)$$

The main observation in this current report is that incubation of intact rat brain mitochondria with tyramine results in suppression of both state 3 and state 5 respiration, accompanied by a rise in mitochondrial PrSSG. These results extend a prior report concerning suppression of MTT reduction by monoamine substrates (Cohen et al., 1997) by demonstrating a direct effect on mitochondrial respiration. This result is not due to the added tyramine per se but rather to a product of MAO activity, because inhibition of MAO activity by a combination of clorgyline and pargyline completely suppressed both PrSSG accumulation and damage to respiratory activity. Because pyruvate was used as substrate, electron flow was initiated at pyruvate dehydrogenase/complex I.

It is estimated that a defect in complex I must exceed 72% to compromise the respiration of nonsynaptic mitochondria (Davey et al., 1998), such as those used in our experiments. Synaptic mitochondria exhibit a lower threshold and show diminished respiration after loss of only 25% of complex I activity. Inhibition of mitochondrial respiration by MAO is attributed to the formation of PrSSG, inhibiting complex I and other thiol-dependent enzymes of the inner membrane (Fig. 5). The magnitude of the effect was similar when respiration was measured directly and when electron flow was measured with MTT (Fig. 3). An implication of the current set of observations is that increased turnover of monoamine neurotransmitters (namely, dopamine, norepinephrine, serotonin, and epinephrine) accompanying a variety of behaviors supported by monoamine neurons could, under appropriate circumstances, feed back and affect respiration within those neurons. In turn, diminished respiration and diminished ATP production could alter neuronal function.

The MTT assay, applied to whole cells, is widely used as an index of cell viability or cell proliferation. However, only a small fraction of MTT reduction can be ascribed to mitochondrial activity (Berridge and Tan, 1993; Liu et al., 1997). With isolated mitochondria, on the other hand, other cellular sites are effectively eliminated and measurements reflect the activity of mitochondria per se, as described in the early experiments of Slater et al. (1963). Berridge and Tan (1993) reported that MTT was not reduced by isolated mitochondria when pyruvate/malate was used as substrate, but succinate was a good substrate. However, their experiments were conducted with bone mitochondria, which may possess special properties. We used pyruvate/malate in the current experiments and observed good reduction of MTT. This observation is in agreement with that of Liu et al. (1997), who studied rat brain mitochondria and found that either pyruvate/malate or succinate can be used.

Parkinson's disease is associated with a 35% decrease in complex I activity in the substantia nigra (Schapira, 1998), the region of the brain that contains the affected dopamine neurons. The defect in Parkinson's disease appears to be intrinsic to the mitochondrial genome as cybrids consisting of ρ° cells without natural mitochondrial DNA, but repopulated with mitochondrial DNA from Parkinson platelets, show a stable 20–25% decrement in complex I activity (Swerdlow et al., 1996; Schapira, 1998). It is of interest that these cybrids show enhanced production of reactive oxygen species and enhanced susceptibility to MPTP compared with cybrids repopulated with mitochondria from control (nonparkinsonian) individuals (Swerdlow et al., 1996). Susceptibility to the damaging effects of MAO-generated H_2O_2 may also be enhanced.

Parkinson's disease is also characterized by a compensatory increase in the turnover of dopamine within surviving dopaminergic neurons (Hornykiewicz and Kish, 1986). Therefore, surviving neurons and adjacent glia are subjected to an oxidative stress emanating from a rise in the steady-state level of H_2O_2, derived from enhanced oxidative deamination of dopamine and its *O*-methylated metabolite, 3-*O*-methyldopamine. This effect should be exacerbated during chronic treatment with L-Dopa, which is decarboxylated in brain to form excess dopamine. Indeed, Przedborski et al. (1993) observed diminished complex I activity in the substantia nigra of rats after long-term treatment with L-dopa.

The current experiments (a) show that mitochondrial respiration initiated with pyruvate can be compromised by MAO-generated H_2O_2 and (b) raise the intriguing question of whether the complex I deficiency that characterizes the parkinsonian brain may be a natural consequence of the disease process itself, induced in sensitive individuals by compensatory increases in dopamine utilization and turnover. Genetic defects have been described in select families of Parkinson's disease patients [namely, the α-synuclein gene and the parkin gene (Polymeropoulos et al., 1997; Kitada et al., 1998)] and in Huntington's disease. How these genes interact with endogenous or environmental factors to produce different mitochondrial lesions and neurodegenerative states is as yet unclear. However, the observations that mitochondrial respiration is specifically at risk as a form of MAO-mediated damage, as described in the current report, and that mixed disulfides linking GSH to protein thiols may play a role open new possibilities for research. The mechanisms that protect against disruption of protein thiol homeostasis require closer scrutiny in mitochondrially based neurodegenerative disease.

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Oxidative Stress, Mitochondrial Respiration, and Parkinson's Disease

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ABSTRACT: **When either oxidizing species, such as H_2O_2 or oxy-radicals, are present in excess or cellular anti-oxidant defenses are lowered, a state of oxidative stress exists. Parkinson's disease is characterized by the loss of dopamine (DA) neurons, which leads to overactivity of the surviving DA neurons and an increase in neurotramsmitter release and turnover. The increased metabolism of DA neurotransmitter by monoamine oxidase (MAO) can be looked upon as an endogenous oxidative stress, leading to damage to Complex I-linked mitochondrial respiration. It remains an open question to what extent the mitochondrial damage seen in Parkinson's disease is of genetic origin and how much is caused by H_2O_2 generated during enhanced turnover of DA, especially during treatment with L-dopa.**

PROLOGUE

It is a pleasure to contribute to this Festchrift volume for Dr. Daniel Gilbert. I first met Dan at a specialty meeting on "Implications of Organic Peroxides in Radiobiology" held at the Argonne National Laboratories, outside of Chicago, in May of 1962. A role for free radicals in biological processes was not in vogue at the time. That awaited the description of superoxide dismutase by Joe McCord and Irwin Fridovich in 1969, and the research and events spawned by that discovery. The prevailing assumption in 1962 was that oxy-radicals were of theoretical interest, but without important roles in biology. The exception was radiobiology because it was clear that the scission of water by high-energy radiation gave rise to superoxide and hydroxyl radicals, associated with tissue damage. But, this was clearly a specialized area of research. The major emphasis at the Argonne conference was on more stable products, such as hydrogen peroxide and organic hydroperoxides.

Such a highly specialized meeting, lying off more-traveled tracks, drew only a slender group of specialists, and a few outsiders, like myself, who had come to listen and learn. Among the key participants were Cheves Walling, well-known for his contributions to the chemistry of free radicals, Dean Burk, who left a lasting mark in enzymatic biochemistry with his Lineweaver-Burk plot, and Frederick Bernheim, who organized and oversaw the "Peroxide Club" that met yearly at the FASEB meeting in Atlantic City. The crucial opening session of the conference laid out the basic

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facts, nuts, bolts, and pieces of chemistry, biology, and physics that go together in approaches to more complex biologic phenomena. With many of the world's experts in attendance, the opening session provided overviews by Walling & Bernheim, and a masterly review by Gilbert on the role of free radicals and peroxides in oxygen toxicity. In the group photograph of the speakers at the meeting (Radiation Research, Supplement 3, 1963), Dan is the "kid" in the first row (FIG. 1). With his grasp of both chemical-physics and biology, and his interest in the important events and personalities in the field, Dan demonstrated a keen knack for providing the audience with the essential facts and keeping them in historical perspective. He is still doing that today with his overview of 50 years of free radical research in this, his own Festschrift volume, sponsored by the New York Academy of Sciences.

As a footnote, it was Frederick Bernheim's wife, Mary, who discovered and characterized tyramine oxidase (Mary L.C. Hare, Tyramine oxidase. I. A new enzyme in liver. Biochem. J. **22:** 968–979, 1928) as part of her doctoral research at Newnahm College in Cambridge (England) at about the time that the visiting Bernheim swept her off her feet and they were married. "Tyramine oxidase" is now better known as monoamine oxidase. It is the subject of the material that follows.

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Front row: Titus C. Evans, B. A. Kihlman, Dean Burk, Hugo Aebi, Paul Kotin, Daniel L. Gilbert, E. L. Powers, Jr., F. H. Sobels, Nicholas A. Milas.

Back row: Elwood V. Jensen, Frederick Bernheim, J. St. L. Philpot, Howard I. Adler, John F. Thomson, Cheves Walling, Orville Wyss, Walter R. Guild.

Kneeling: Robert N. Feinstein.

Missing from photograph: Raymond Latarjet, John H. Pomeroy, Bernard Smaller.

FIGURE 1. Reproduced with permission of Academic Press.

INTRODUCTION

A state of oxidative stress exists when either oxidizing species, such as H_2O_2 or oxy-radicals, are present in excess, or cellular antioxidant defenses are lowered. Oxidative stress drives cellular systems to an oxidized state. One cellular target is the redox state of protein sulfhydryls (Pr-SH). Many enzymes contain essential thiol groups, which are derived from cysteine residues and are essential for biologic function. When these thiols are blocked by disulfide formation, such as protein-glutathione mixed disulfides (PrSSG), enzymatic function is suppressed. This change represents an oxidative step that is reversible catalyzed by enzymes such as thioredoxins and protein disulfide isomerase.[1,2]

One factor that markedly affects the redox state of protein thiols is the removal of H_2O_2 via the enzyme glutathione (GSH) peroxidase (Eqn 1). Detoxification of H_2O_2 results in the formation of glutathione disulfide (GSSG). In turn, GSSG reacts with protein thiols to form protein mixed disulfides (Eqn. 2).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \quad (1)$$

$$GSSG + PrSH \rightarrow PrSSG + GSH \quad (2)$$

Parkinson's disease is characterized by the loss of dopamine (DA) neurons from the substantia nigra, a heavily pigmented region of the human brain. The pigment is derived from DA itself and therefore loss of DA neurons is accompanied by a loss of pigmentation in this region of the brain. The surviving DA neurons are overactive. They increase their release of neurotransmitter and turnover of DA in a compensatory manner.[3] Indeed, this compensation effectively delays overt motor abnormalities until the loss of DA neurons is 80% or greater.

Most of the released DA is normally recaptured by the presynaptic nerve terminal and stored in vesicles, permitting reutilization of the neurotransmitter. However, a portion of the DA does not reach the safety of the storage vesicles, but instead encounters mitochondria, which are the site of metabolism of monoamine neurotransmitters by the enzyme monoamine oxidase (MAO). The metabolic turnover of DA closely parallels neuronal activity and increases as neuronal activity increases. This is evident from the rise in the acid metabolites of DA, namely DOPAC and homovanillic acid (HVA), in brain, which can be seen at autopsy in Parkinson's disease[3] and in animal models. MAO itself is an H_2O_2-generating enzyme (Eqn. 3). Therefore, the increased turnover of DA neurotransmitter and its O-methylated metabolite (3-O-methyl-DA) can be looked upon as an endogenous oxidative stress, increasing the steady state level of H_2O_2 and evoking oxidation of GSH to GSSG both pre- and post-synaptically in the region of DA neurons and nerve terminals.

$$\text{tyramine} + O_2 + H_2O \rightarrow H_2O_2 + NH_3 + p\text{-hydroxyphenylacetaldehyde} \quad (3)$$

It was previously demonstrated that oxidation of DA and other monoamines (viz., tyramine, benzylamine) by isolated brain mitochondria can detrimentally affect mitochondrial electron transport.[4] Damage to electron transport, measured by a dye-reduction method, was evident when either pyruvate or succinate was used as the mitochondrial substrate. However, damage was more severe when pyruvate was used, compared to succinate. Pyruvate initiates electron flow at Complex I, while succinate initiates electron flow at Complex II. In more recent experiments, we measured

directly the oxygen consumption (respiration) associated with electron flow. These experiments[5] showed that respiration is impaired by MAO activity and that this change is accompanied by a marked accumulation of mitochondrial PrSSG.

Parkinson's disease is also characterized by a significant loss (~35%) of Complex I activity from mitochondria.[6] Complex I takes reducing equivalents from pyruvate dehydrogenase and transfers them to coenzyme Q of the respiratory chain. Complex I activity is dependent upon protein thiol groups[7] and therefore, it can be affected by changes in the thiol redox state of associated proteins. Similarly, pyruvate dehydrogenase is also SH-dependent.[8] In the study described here, MAO activity evoked both a rise in PrSSG and inhibition of pyruvate-linked mitochondrial respiration.[5]

METHODS

Mitochondria were isolated from the pooled whole brain (less the cerebellum) of groups of three Sprague-Dawley rats (250–275 g) by a minor modification of the method of Clark and Nicklas.[9] The isolation medium consisted of 5 mM Mops, containing 0.225 M mannitol, 0.075 M sucrose, and 1.0 mM EGTA, adjusted to pH 7.4 with KOH. Isolation was carried out in the cold with a refrigerated Sorvall RC24 centrifuge, equipped with an SS 34 rotor for 10 min at 15,000 × *g*. The isolated mitochondria were suspended in cold Mops buffer at a concentration of 15–20 mg mitochondria protein/ml and maintained in an ice bath until used. The yield was 3–4 mg mitochondrial protein per rat brain.

Incubations were conducted by dilution of an aliquot of the mitochondrial preparation to 0.5 mg or 1.0 mg mitochondrial protein/ml in the respiration buffer (pH 7.2), which consisted of 5 mM Hepes, 125 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, and 1 mM $MgCl_2$ with 0.5 mg bovine serum albumin/ml at 27°C. Incubations were carried out at 27°C in a volume of 1 ml in plastic tubes (12-ml) on a water bath with gentle shaking for 15 min. Samples were processed individually with immediate assessment of respiration and rotation among the experimental groups. Samples not incubated with MAO inhibitors (2 μM clorgyline plus 2 μM pargyline) received additions of the inhibitors after the incubation was complete, just prior to the measurement of respiration or electron flow. Each experiment consisted of 10–12 samples (3–4 samples per group).

Respiration was measured in a miniature chamber system (0.6 ml capacity; Instech Labs. Plymouth Meeting, PA.), equipped with a magnetic stirrer and maintained at 27°C. Oxygen consumption was assessed with a YSI Model 5300 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH). Measurements were made sequentially after the addition of pyruvate/malate (5 mM each) (State 4 respiration), followed by 0.4 mM ADP (State 3) and, lastly, 10 μM FCCP (State 5). In some experiments, ADP was omitted and State 5 respiration was measured directly. Respiratory activity of the stock mitochondrial preparation, which was held on ice, was well maintained and did not change over the course of 3–4 h.

PrSSG was measured with a modification of the method of Akerboom and Sies.[10] The liberated GSH was measured on a plate reader with a modification of the enzymatic recycling method of Tietze.[11] Protein was measured by the method of Lowry

TABLE 1. Effect of MAO activity on mitochondrial state 3 and state 5 respiration

Respiratory state	natoms oxygen/min/mg mitochondrial protein (% untreated control)			
	Without MAO inhibitors		With MAO inhibitors	
	Control	Tyramine	Control	Tyramine
State 3	100.0 ± 3.3	67.2 ± 1.7[a]	108.8 ± 3.8	95.2 ± 3.7[b]
State 5	100.0 ± 2.5	59.9 ± 1.9[a]	100.3 ± 2.7	99.5 ± 2.9[b]

NOTE: Isolated rat brain mitochondria were incubated with 500 μM tyramine for 15 minutes at 27°C and, subsequently, State 3 and State 5 respiration were measured with an oxygen electrode and with 5 mM pyruvate plus 5 mM malate as substrate. Where indicated, the MAO inhibitors clorgyline and pargyline (2 μM each) were present. Data are the mean ± SEM for N = 5–6 per group for State 3 respiration and N = 10–11 for State 5 respiration .

[a] $p < 0.001$ vs. untreated control.

[b] $p < 0.001$ vs. tyramine without MAO inhibitors.

et al.[12] and was used to normalize data for both PrSSG and respiration, which were expressed per mg protein. Data are expressed as the mean ± SEM. Statistical assessment was conducted by the Tukey-Kramer multiple comparison test or, where appropriate, by the 2-tailed Student *t*-test.

RESULTS

Prior to conducting experiments, the preparations of rat brain mitochondria were characterized. The respiratory control ratios (State 3/State 4) of freshly isolated mitochondria were in the range 5.5–7.0 with 5 mM pyruvate plus 5 mM malate as substrate. State 3 respiration was in the range 72–103 ng-atoms oxygen/min/mg protein. These parameters are in good agreement with reports in the literature.[9]

Rat brain mitochondria were exposed to 500 μM tyramine for 15 min at 27°C in either the absence of presence of MAO inhibitors. The concentration of tyramine corresponds to the level of neurotransmitter in the cytosol of catecholamine neurons

TABLE 2. Effect of MAO activity with either tyramine or dopamine as substrate on mitochondrial levels of protein-glutathione mixed disulfides (PrSSG)

Time (min)	mitochondrial PrSSG (μ moles/mg protein)		
	Control	Tyramine	Dopamine
0	0.03 ± 0.02	0.03 ± 0.02	0.03 ± 0.02
7.5	0.05 ± 0.01	0.53 ± 0.03	0.41 ± 0.03
15	0.07 ± 0.01	0.84 ± 0.05	0.65 ± 0.01
22.5	0.05 ± 0.02	0.94 ± 0.06	0.77 ± 0.04

NOTE: Isolated rat brain mitochondria were incubated with either 500 μM tyramine or 500 μM dopamine for 22.5 minutes at 27°C. Subsequently a protein pellet was isolated with perchloric acid and the level of PrSSG was measured. Data are the mean ± SEM for $N = 4$. All elevations induced by tyramine or dopamine were significant ($p < 0.001$).

(discussed in Cohen, Farooqui & Kesler[4]). The results of these experiments (TABLE 1) show that State 3 respiration was suppressed by 32.8% and State 5 respiration by 40.1% after exposure to tyramine. Tyramine is a mixed MAO-A/MAO-B substrate. Inhibition of both isoforms of MAO with a mixture of 2 μM clorgyline (selective MAO A inhibitor) and 2 μM pargyline (selective MAO B inhibitor) prevented the damage to mitochondrial respiration (TABLE 1). These observations are consistent with damage by H_2O_2, which is a product of MAO activity. Because pyruvate was used as substrate, electron flow was initiated at pyruvate dehydrogenase/ Complex I.

Mitochondria were also evaluated for the effects of the incubation conditions alone. This was done because it is well known that isolated mitochondria are susceptible to damage by agitation, particularly at 37°C. Indeed, most investigations of respiration are carried out at lower temperature, such as 30°C or room temperature. We used 27°C in order to limit damage by environmental conditions. Simply shaking the mitochondria at 27°C decreased respiration by 27.3% ± 0.7% for State 3 and 21.7% ± 1.5% for State 5 ($p < 0.01$, n = 5/group, 2 experiments). However, the data in TABLE 1 are expressed as the effects of tyramine relative to incubated controls and, therefore, changes due to experimental conditions, unrelated to tyramine, cancel out. Nonetheless, the loss of a highly vulnerable fraction of respiratory activity may cause the effect of tyramine to be underestimated in TABLE 1. The stock, concentrated suspension of mitochondria (15–20 mg protein/ml) in Mops buffer, held on ice, was stable and did not lose respiratory activity over the course of the experiments (3–4 h).

The results shown in TABLE 2 show that the levels of PrSSG rose rapidly during the first 7.5 minutes of incubation. The rise was greater than 10-fold. Further accumulation of PrSSG was seen at 15 minutes and 22.5 minutes. In separate experiments it was observed that inhibition of MAO by a mixture of clorgyline and pargyline suppressed the rise in PrSSG: while PrSSG achieved levels of 0.4 to 1.2 μmoles/ mg protein with either tyramine or dopamine, the MAO inhibitors suppressed PrSSG to control levels (less then 0.04 μmoles/mg protein). As in the study of respiration, this result was not due to the added tyramine per se, nor to possible effects of autoxidizing DA, because MAO inhibitors completely suppressed the rise in PrSSG.

DISCUSSION

The main observations are that incubation of intact rat brain mitochondria with tyramine results in suppression of both State 3 and State 5 respiration, accompanied by a rise in mitochondrial PrSSG. The result is not due to the added tyramine per se, but rather, to a product of MAO activity, because inhibition of MAO activity by a combination of clorgyline and pargyline completely suppressed both PrSSG accumulation and damage to respiratory activity. Because pyruvate was used as substrate, electron flow was initiated at pyruvate dehydrogenase/Complex I.

Mitochondrial defects associated with Complexes I–IV of the respiratory chain occur in a number of neurodegenerative diseases, including Parkinson's disease, Huntington's disease, Friedreich's ataxia, hereditary spastic paraplegia, Alzheimer's

disease, and amyotrophic lateral sclerosis.[13,14] Moreover, several animal models of neurodegenerative disease are based on mitochondrial toxins, such as MPTP, which inhibits Complex I of the electron transport chain, producing an animal model for Parkinson's disease, or 3-nitroproprionic acid and malonate, which inhibit Complex II, producing models for Huntington's disease.[15] Therefore, mitochondrial defects appear to play primary roles in disease expression and progression. Defects in cellular respiration lead to diminished ATP production, increased sensitivity to oxidative stress and, eventually, to apoptotic or necrotic neuronal cell death.[16] The defect in Parkinson's disease is localized to Complex I and is seen at autopsy as a 35% decrement in the substantia nigra, which is the region of the brain containing the affected DA neuron cell bodies.

Mitochondrial respiratory defects can be directly inherited or may be acquired as the result of exposure to stressors. The experiments described here identify a mitochondrial enzyme, monoamine oxidase, and the turnover of monoamine neurotransmitters by MAO, as a source of oxidative stress that can suppress mitochondrial respiration. MAO is a flavo-enzyme, localized to the outer mitochondrial membrane.[17] It plays an essential metabolic role in the turnover of dopamine, serotonin, norepinephrine, and epinephrine in the central nervous system. As discussed earlier, oxidative deamination of monoamines by MAO is accompanied by the reduction of molecular oxygen to H_2O_2,[18,19] a potentially toxic agent that can evoke changes in the cellular thiol status, as well as direct damage to mitochondrial DNA.

H_2O_2 is also formed naturally during mitochondrial respiration. It is estimated that 1–3% of consumed oxygen is converted to H_2O_2.[20] H_2O_2 that "leaks" from the electron transport chain damages both mitochondrial proteins and mitochondrial DNA.[21,22] It is widely believed that the H_2O_2 generated in this way is responsible for the decline in mitochondrial function in aging, reperfusion injury, and certain disease states.[23,24] However, the quantity of H_2O_2 generated by mitochondrial MAO exceeds by a wide margin the amount generated during electron flow. Hauptmann *et al.*[19] studied the oxidation of 2 mM tyramine by rat brain mitochondria and reported that H_2O_2 production was 48-fold greater than that from succinate during electron transport in the presence of antimycin A. Hence, MAO possesses a considerable toxic potential. Moreover, the mitochondrial localization of MAO makes this enzyme uniquely situated to evoke selective mitochondrial damage.

It remains an open question how much of the mitochondrial damage seen in Parkinson's disease is of strictly genetic origin and how much is derived from damage by H_2O_2 generated during enhanced turnover of DA, particularly during treatment with L-dopa. Moreover, it remains to be seen whether or not mitochondrially generated H_2O_2 interacts with genetic factors in subjects predisposed to Parkinson's disease. For example, recent studies have described genetic defects, such as the alpha synuclein gene and the parkin gene in select families with Parkinson's disease[25,26]; other defects affecting Complex II of the respiratory chain have been described in Huntington's disease. How these genes interact with endogenous or environmental factors to produce different mitochondrial lesions and neurodegenerative states is as yet unclear. For Parkinson's disease, the production of H_2O_2 during the natural turnover of DA, or the enhanced turnover associated with overt symptomatology, may place genetically susceptible subjects at risk for damage to mitochondrial respiratory activity. If such events are mediated by PrSSG formation, they may be reversible.

Therefore, further studies of the relationships between MAO activity, PrSSG accumulation, and defects in mitochondrial respiration are clearly warranted.

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Chapter 24

Oxidative Stress and Parkinson's Disease

Gerald Cohen

1. CHARACTERISTICS OF PARKINSON'S DISEASE

Parkinson's disease (PD) is a progressive neurodegenerative disorder affecting primarily the dopamine (DA) neurons that arise in the midbrain (mesencephalon) and project to the putamen and caudate regions (the striatum) of the brain, areas concerned with the control of motor movements (Hornykiewicz and Kish, 1986). Unaffected (or minimally affected) by the disease are DA neurons that arise in the midbrain and project to cortical and limbic regions; overactivity of the latter neuronal circuits has been implicated in schizophrenia (e.g., Lozoncy *et al.*, 1987). Also unaffected are other monoaminergic neurons, specifically the norepinephrine (NE)-secreting and serotonin-secreting neurons of the brain.

The DA neurons that degenerate in PD arise in the substantia nigra, a local region of the midbrain. As its name implies, this region is normally heavily pigmented; the pigment is readily visible to the unaided eye as a brown-to-black region of the mesencephalon. The pigment is an insoluble polymer that is related to melanin of skin, and has been termed *neuromelanin*. However, unlike skin, the pigment is not derived by enzymatic synthesis from L-dopa catalyzed by tyrosinase, because tyrosinase is absent from the brain. Rather, neuromelanin appears to be formed via a slow, nonenzymatic process based on the autoxidation and spontaneous polymerization of the catecholamine DA (Graham, 1978). As with melanin of skin, quinoidal intermediates react with soluble tissue thiols, such as glutathione and cysteine, to incorporate sulfur residues into the matrix of the polymer (Carstam *et al.*, 1992).

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A hallmark of PD is the disappearance of pigmentation from the substantia nigra. This is readily apparent visually to neuropathologists at autopsy and, because of this, absence of neuromelanin became an early marker for PD. It is now known that the marked diminution or absence of pigmentation does not represent a change in the chemical process that forms neuromelanin. Rather, loss of pigment reflects the loss of DA neurons via an underlying neurodegenerative process. The key findings that prove this point are: (1) a loss of the neurotransmitter DA and (2) loss of the biosynthetic enzyme tyrosine hydroxylase from autopsy specimens of brain. These two markers reflect loss of DA-secreting neurons. With the degeneration and loss of DA neurons, the neuromelanin associated with the cell bodies is phagocytized and disappears.

The reasons for the selective loss of nigrostriatal DA neurons in PD remain obscure. Current thoughts and research center around possible genetic predisposition, environmental factors, and an oxidative stress that may be derived directly from the natural utilization and turnover of the neurotransmitter DA. A recent conference stressed an interplay between all three factors (Gorrell *et al.*, 1996). At least one clinical trial has been directed toward attempting to slow progression driven by DA turnover or driven by exposure to putative environmental toxins (Parkinson Study Group, 1989a). Although intervention with the monoamine oxidase (MAO) B inhibitor deprenyl (selegiline) gave evidence for slowing of disease progression in subjects with early PD (Parkinson Study Group, 1989b), these results remain the subject of some debate. In clinical trials, disease progression is most frequently assessed from behavioral scores; however, to properly assess motor function, experimental drugs need to be first washed out, lest drug effects interfere with the assessment of motor scores. In the deprenyl trial (the so-called DATATOP study) a mild symptomatic effect was detected, which could have been responsible, in part, for the behavioral benefit of deprenyl. Considerable drug benefit was retained after washout, but disease progression was still evident (Parkinson Study Group, 1993; Olanow *et al.*, 1995). The costly and newly developed technique of PET scanning (positron emission tomography) can provide more direct assessment of changes to nigrostriatal neurons that are affected in PD.

Generally, the expression of symptomatology (tremor, akinesia) in PD is delayed until 80% or more of the nigrostriatal DA neurons have been lost. Progression with further loss of DA neurons occurs despite treatment, which is mainly directed at controlling symptomatology, i.e., to enhancing dopaminergic neurotransmission. Widely used treatments consist of L-dopa (to enhance brain synthesis of DA), deprenyl (to impede metabolism of DA), and DA agonists (to function in place of the missing DA). Considerable interest exists in identifying factors that contribute to disease progression because such information could lead to the development of new treatment approaches to curtail the progressive loss of DA neurons.

2. DOPAMINERGIC NEUROTOXINS

Concepts of oxidant stress in PD have been driven by experiences with two dopaminergic neurotoxins, namely, 6-hydroxydopamine (6-OHDA; 2,4,5-trihydroxyphenylethylamine) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Both of these agents have been used experimentally to produce animal models of PD in which nigrostriatal

DA neurons are lesioned relatively selectively. Studies on the roles of superoxide and hydroxyl radicals in the mechanism of action of 6-OHDA played an important historical role in the early development of concepts of oxy-radical toxicity in biological systems (e.g., Cohen and Heikkila, 1974; Cohen *et al.*, 1976).

2.1. 6-Hydroxydopamine

6-OHDA was first discovered as a neurotoxic agent by Tranzer and Thoenen in the 1960s as an agent that disrupted brain slices that were prepared for electron microscopic examination of the vesicular binding properties of catecholamine analogues. It was subsequently determined that the agent produced relatively selective damage to peripheral sympathetic neurons (NE-secreting) when injected peripherally, but that it did not cross the blood–brain barrier (reviewed by Kostrzewa and Jacobowitz, 1974). However, injections into the cerebral ventricles or directly into target brain tissue (e.g., the substantia nigra or striatum) have been widely used to lesion targeted DA or NE neurons of the brain.

As a hydroxylated DA analogue, 6-OHDA is recognized by the catecholamine transporters present in the axonal membranes of catecholamine neurons and gains access to the neurons via this pathway (Kostrzewa and Jacobowitz, 1974). Axonal transport targets catecholamine neurons and explains the extraordinary specificity of neurotoxic action. Under the appropriate experimental conditions, only catecholamine neurons are detrimentally affected, while other neuronal types are spared. The use of selective inhibitors of either the DA transporter or the NE transporter can provide further experimental specificity to selectively spare either DA or NE neurons.

6-OHDA is highly unstable in aqueous solution at neutral pH and in the presence of oxygen. The reaction with molecular oxygen yields hydrogen peroxide (H_2O_2) and a red quinone [Eq. (1)]. The rapid formation of red quinoidal products over 10–20 s at pH 7.4 is readily apparent visually. This observation led many early workers to add ascoroic acid as a reducing agent to "protect" the 6-OHDA, i.e., to prevent discoloration of the solution containing 6-OHDA. However, the situation is more complicated and ascorbate plays a more prominent role as an agent that exacerbates the neurotoxic effects of 6-OHDA. Entry into catecholamine neurons requires that 6-OHDA be present in its reduced form, which is recognized by catecholamine transporters. Hence, ascorbate helps to maintain 6-OHDA in a transportable form. However, the same role is served by endogenous ascorbate (e.g., in blood plasma). Indeed, direct intravenous injection of 6-OHDA-quinones leads to the same destruction of peripheral sympathetic neurons as 6-OHDA (Heikkila *et al.*, 1973). Tissue ascorbate also promotes toxicity by recycling the quinones [Eq. (2)].

$$\text{6-OHDA} + O_2 \xrightarrow{\text{autoxidation}} o\text{- and } p\text{-quinones} + H_2O_2 \tag{1}$$

$$\text{ascorbate} + \text{quinone} \rightarrow \text{dehydroascorbate} + \text{6-OHDA} \tag{2}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \tag{3}$$

The H_2O_2 generated in Eq. (1) can lead to damage of DA neurons (Heikkila and Cohen, 1971); available evidence points to the formation of hydroxyl radicals [Eq. (3)] as neurotoxic intermediates (Cohen and Heikkila, 1974; Cohen *et al.*, 1976). The quinones

can express toxicity via the formation of adducts with protein -SH groups (Graham *et al.*, 1978) leading to inactivation of enzymatic or structural properties of key proteins. Although both H_2O_2- and quinone-based mechanisms are probably operative, the available evidence favors the view that H_2O_2 plays the more dominant toxic role. For example, ascorbate added to tissue slices prevents the appearance of colored quinones, but potentiates neurotoxicity (Heikkila and Cohen, 1972). The reason for this effect is that ascorbate recycles the quinones by reduction [Eq. (2)] and, thereby, amplifies H_2O_2 production [Eq. (1)]. Indeed, in experiments carried out with tissue slices, formation of H_2O_2 by relatively small amounts of 6-OHDA was controlled by the level of ascorbate. In addition, analogues of 6-OHDA with methyl groups blocking the ring positions that would normally make adducts with -SH groups of proteins, still exhibit neurotoxicity (Graham *et al.*, 1978).

The generation of superoxide by 6-OHDA was detected in experiments with superoxide dismutase (Heikkila and Cohen, 1973). By scavenging superoxide radicals, superoxide dismutase suppressed both the formation of quinones and the accumulation of H_2O_2. Thus, although superoxide dismutase catalyzes a reaction that generates H_2O_2 (namely, the dismutation of superoxide to yield H_2O_2 and oxygen), its net effect is to suppress the overall formation of H_2O_2 and, subsequently, the derived hydroxyl radicals. The explanation for this apparently paradoxical situation can be found in the reaction mechanism for autoxidation of 6-OHDA, where Q stands for 6-OHDA-quinone, SQ· is the corresponding semiquinone, and $\cdot O_2^-$ is the superoxide radical anion:

$$\text{6-OHDA} + O_2 \rightarrow \text{SQ}\cdot + \cdot O_2^- + H^+ \qquad \text{(slow)} \tag{4}$$

$$\text{6-OHDA} + \cdot O_2^- + H^+ \rightarrow \text{SQ}\cdot + H_2O_2 \qquad \text{(fast)} \tag{5}$$

$$\text{SQ}\cdot + O_2 \rightarrow \text{Q} + \cdot O_2^- \qquad \text{(fast)} \tag{6}$$

The direct reaction of oxygen with 6-OHDA is relatively slow [Eq. (4)] whereas the oxidation of 6-OHDA by superoxide is much faster [Eq. (5)]. After initiation of the reaction sequence [Eqs. (4) and (5)], SQ· reacts rapidly with molecular oxygen to regenerate superoxide [Eq. (6)]. Equations (5) and (6) establish a radical chain reaction in which superoxide is consumed as it oxidizes 6-OHDA and then it is regenerated; the reaction chain bypasses the slow step [Eq. (4)] and promotes formation of H_2O_2 [Eq. (5)] and quinones [Eq. (6)]. The chain reaction can be intercepted by superoxide dismutase, or by catecholamines (such as DA or NE) which are also oxidized by superoxide (Sachs *et al.*, 1975; Cohen and Heikkila, 1977). In this sense, the catecholamines act as scavengers for superoxide, suppressing the overall rate of production of H_2O_2 and quinones by replacing the extraordinarily rapid autoxidation sequence for 6-OHDA with the much slower reactions for catecholamine autoxidation at neutral pH.

The neurotoxic analogue 6-aminodopamine behaves differently. Its autoxidation rate is not catalyzed by superoxide (Sachs *et al.*, 1975; Cohen and Heikkila, 1977). In experiments conducted *in vivo*, 6-OHDA and 6-aminodopamine were differentially affected by endogenous catecholamines, consistent with their relative dependence on (6-OHDA) or independence (6-aminodopamine) from superoxide as a catalyst: Prior depletion of NE (with α-methyl-*p*-tyrosine methyl ester) potentiated the neurotoxicity of 6-OHDA, but not 6-aminodopamine. Enhancing the level of NE protected against 6-OHDA, but not 6-aminodopamine. However, elevated tissue levels of octopamine (a

phenolic monoamine that does not scavenge superoxide) failed to protect against either 6-OHDA or 6-aminodopamine neurotoxicity. These results are in keeping with the ability of NE in sympathetic neurons *in vivo* to suppress the catalytic formation of peroxide by 6-OHDA, but not 6-aminodopamine.

The hydroxyl radical (·OH) is generated from H_2O_2 by the Fenton reaction with iron in the ferrous form [Eq. (3)]. Hydroxyl radicals can lead to generalized cellular damage based on the oxidation of membrane lipids (lipid peroxidation), enzymes, structural proteins, and nucleic acids. Hydroxyl radicals were detected *in vitro* during the autoxidation of 6-OHDA and 6-aminodopamine (Cohen and Heikkila, 1974). Peripheral sympathetic nerves innervating the heart and iris were protected from neurodegeneration *in vivo* by scavengers of ·OH (Cohen *et al.*, 1976). Jonsson (1976) showed that ·OH scavengers did not inhibit the neuronal accumulation of [^{3}H]-6-OHDA, thereby confirming that protection was the result of interruption of the cytotoxic mechanism, and not an effect on axonal transport of 6-OHDA.

6-OH-dopa (2,4,5-trihydroxyphenylalanine), the amino acid analogue of 6-OHDA, is also neurotoxic, but exhibits some unusual properties. 6-OH-dopa readily crosses the blood–brain barrier and achieves access to central catecholamine neurons. Neurotoxicity requires prior conversion to 6-OHDA. The available evidence indicates that transformation to 6-OHDA takes place within catecholamine neurons and does not require the catecholamine transporter (Evans and Cohen, 1993). However, the unusual aspect is that 6-OH-dopa selectively targets NE-secreting neurons in the CNS, while sparing DA neurons (Kostrzewa and Jacobowitz, 1974). DA neurons are spared even though they accumulate quite high levels of 6-OHDA (Evans and Cohen, 1989) approaching those of endogenous DA (which are in the range of 50 mM). These observations indicate that central DA neurons are endowed with special protective mechanisms compared with NE neurons. The selectivity of 6-OH-dopa is opposite to that of MPTP (see below), which targets DA neurons but spares NE neurons.

2.2. MPTP

The MPTP story opened with a single case report in 1979 by a group of investigators at the National Institutes of Health (NIH). This important observation encountered difficulty in gaining access for publication, but was eventually published in the inaugural volume of *Psychiatry Research* (Davis *et al.*, 1979). Autopsy of a young adult who had overdosed on a narcotic drug showed extensive damage to the substantia nigra paralleling a motor defect resembling PD. Because PD is a disease of aging, loss of nigral DA neurons in a young adult was unusual. The subject had been in the habit of preparing his own "designer" drugs by organic synthesis and he kept a notebook with details. It was discovered that during the preparation of a meperidine analogue via a reverse synthesis pathway, he had used heat to speed a reaction and this had resulted in an unexpected chemical elimination of a side chain (dealkylation) with the resultant formation of a double bond in the 4–5 position of a piperidine ring. Other products were also formed, but the main product, MPTP, was considered to be the culprit for rapid onset of parkinsonism in a young adult. This tentative explanation was confirmed in 1983 in a publication in *Science* (Langston *et al.*, 1983). This second publication extended the observations to a broader series of young adults who had inadvertently been exposed to

MPTP as a contaminant in illicit drug preparations. The latter study strengthened considerably the link to MPTP, opening a new prospect for understanding PD. MPTP toxicity in human subjects is viewed as a chemically induced form of parkinsonism.

Unlike research on 6-OHDA, which was facilitated by the rapid response of rodents (rats, mice) to the injected neurotoxin, research into the mechanism of action of MPTP was severely hampered by an absence of animal models. Initial studies by the group at the NIH who made the first report drew a blank in studies with rats, mice, rabbits, and cats. It was not until monkeys were tested that symptoms of PD paralleling overt loss of central DA neurons appeared with relatively low doses of MPTP, commensurate with the doses to which human drug users had been exposed (Burns *et al.*, 1983). However, with only monkeys as an *in vivo* model, research in this important area was severely constrained until other investigators showed that mice could be used when the dose of MPTP was raised about 100-fold (Heikkila *et al.*, 1984; Hallman *et al.*, 1985). In addition, an important new tool, mesencephalic cell cultures (Mytilineou and Cohen, 1984), was added to the research armamentarium; DA neurons in cell cultures proved to be exquisitely sensitive to MPTP.

It has been established that the neurotoxicity of MPTP is directed relatively selectively at the dopaminergic nigrostriatal tract, while DA neurons in the limbic system, as well as central NE and serotonin neurons, are spared. Biochemical and histological studies verified the destruction of DA cell bodies in the substantia nigra and the loss of dopaminergic innervation in the caudate and putamen.

The neurotoxic mechanism involves the conversion of MPTP, which is a protoxin, to 1-methyl-4-phenylpyridinium (MPP^+), which is the ultimate toxin. Initial studies with mesencephalic cultures had implicated MAO, because deprenyl or pargyline (MAO-B inhibitors) protected the DA neurons (Mytilineou and Cohen, 1984). One possibility was that the MAO inhibitors protected by suppressing the metabolism of DA, which produces H_2O_2 as an end product. However, studies by Chiba *et al.* (1984) with isolated mitochondria established the link to MAO as transformation of MPTP to MPP^+ [Eq. (7)]. The form of MAO required to transform MPTP is MAO-B. *In vivo*, inhibitors of MAO-B, such as deprenyl (Cohen *et al.*, 1984) or pargyline (Langston *et al.*, 1984), prevent the destruction of nigrostriatal neurons in monkeys.

$$\text{MPTP} \xrightarrow{\text{MAO-B}} \text{MPDP}^+ \xrightarrow{\text{spontaneous}} \text{MPP}^+ \tag{7}$$

There were several surprising aspects to this story. First, MAO, which normally deaminates substrates, was invoked for a dehydrogenation reaction to yield a double bond. Second, the transformation took place in astrocytes, which are rich in MAO-B, but astrocytes were not themselves damaged. Third, the product, the aromatic amine MPP^+, was recognized by the dopamine transporter, resulting in a marked accumulation of the toxin by DA neurons (Javitch *et al.*, 1985). In this latter regard, susceptibility to both 6-OHDA and MPP^+, and the targeting of vulnerable neuronal types, is based on accumulation of the neurotoxin mediated by the axonal membrane transporter systems for catecholamines.

The toxic effects of MPP^+ require an accumulation by mitochondria where electron transport is inhibited at the level of complex I (Nicklas *et al.*, 1985; Ramsay *et al.*, 1986). Poisoning of the electron transport chain is responsible for the eventual demise of

nigrostriatal DA neurons. Spurred by the latter findings, yet another surprising fact was uncovered, namely, that PD appears to be characterized by a similar defect at the level of Complex I in the substantia nigra, but not in brain regions unaffected by the disease (e.g., Schapira *et al.*, 1990). This observation has given rise to concepts that PD may be caused by environmental mitochondrial toxins, perhaps related to MPTP (e.g., Mizuno *et al.*, 1995; Gorrell *et al.*, 1996).

Inhibition of mitochondrial electron transport (e.g., by rotenone or antimycin A) leads to increased release of reactive oxygen species, such as superoxide and H_2O_2, from mitochondria (Zoccarato *et al.*, 1988). In turn, these agents may spur cell death. Thus, MPTP toxicity may derive from an oxidative stress. In experiments with isolated mitochondria, Cleeter *et al.* (1992) observed that inactivation of Complex I by directly added MPP^+ required the presence of oxygen, in keeping with an oxidative mechanism. Moreover, protection was observed with added antioxidants (ascorbate, glutathione); catalase, a specific enzymatic scavenger of H_2O_2, was also protective. These observations point to an oxidative mechanism for inhibition of Complex I. Chiueh *et al.* (1992) used the salicylate trapping method and *in vivo* dialysis to detect hydroxyl radicals in the caudate nucleus during exposure to MPP^+. Because MPP^+ causes release of DA from neurons, the authors suggested that either DA autoxidation or its oxidative deamination by MAO may play a role in ·OH formation by MPTP/MPP^+ *in vivo*. It is of interest that transgenic mice with increased Cu/Zn-superoxide dismutase are resistant to the neurodegenerative effects of MPTP (Przedborski *et al.*, 1992). These observations, as a whole, indicate a prominent role for oxy-radicals and oxidative stress in parkinsonism induced by MPTP.

3. OXIDATIVE STRESS AND PARKINSON'S DISEASE

A state of oxidative stress exists when either oxidizing species (e.g., oxy-radicals, peroxides) are present in excess, or cellular antioxidant defenses are lowered. Oxidative stress drives cellular systems to an oxidized state. An increased metabolic demand by various cellular support or repair mechanisms can be detrimental to other biological needs. Many markers are available to detect oxidative changes in cellular systems, including lipid peroxidation, formation of protein carbonyls, loss of reducing substances (such as GSH), changes in antioxidant enzymes, and oxidative damage to DNA. Over the years considerable evidence has been amassed from autopsy studies and from experimental studies with animal models that point to the presence of an oxidant stress associated with PD (e.g., Jenner, 1991; Fahn and Cohen, 1992; Ames *et al.*, 1993). A number of authors have addressed the issue that oxidative stress and oxidative damage may play a critical role in neurodegenerative diseases in general, including PD (see Ames *et al.*, 1993; Coyle and Puttfarcken, 1993; Cohen and Werner, 1994; Beal, 1995).

3.1. The L-Dopa Question

From the outset it must be noted that many observations made on autopsy specimens of brain from parkinsonian subjects are partially compromised because most subjects were in treatment with L-dopa. L-Dopa autoxidizes slowly (Basma *et al.*, 1995) to form reactive quinones and H_2O_2. The process is similar to that described in Eqs. (4)–(6) and

Eq. (3), but at a much slower overall rate. In this process, oxy-radical intermediates, such as superoxide, hydroxyl, and semiquinone free radicals, are generated. Hence, the question arises: Does the appearance of markers of oxidative stress in autopsy specimens reflect the disease process itself or does it reflect a side effect of treatment with L-dopa?

The issue is complex and data are available to support both points of view. First, a limited number of observations have been made on patients not treated with L-dopa, which support the presence of an oxidative stress unrelated to treatment. Second, patients treated with L-dopa show better survival, and there is no direct evidence for toxicity of chronic L-dopa in animals models (Hefti *et al.*, 1980). On the other hand, L-dopa exhibits toxicity in cell culture experiments, directed relatively specifically at DA neurons (Olney *et al.*, 1990; Mytilineou *et al.*, 1993; Mena *et al.*, 1993). And, in two *in vivo* models, albeit under special experimental circumstances, evidence for detrimental effects of L-dopa on survival of DA neurons has been presented (Steece-Collier *et al.*, 1990; Blunt *et al.*, 1993). Adding fuel to the fire is the observation that chronic administration of L-dopa to rats produces a Complex I defect in dopaminergic regions of brain (Przedborski *et al.*, 1993). Supporting an opposite point of view is the observation that exposure of mesencephalic cultures to L-dopa induces a compensatory rise in GSH, which prevents a loss in cell viability during exposure to an organic hydroperoxide (Han *et al.*, 1996). Thus, the observed effects of L-dopa are double-edged, and it remains unclear whether L-dopa "therapy" carries a toxic potential, perhaps in selected subsets of PD patients.

3.2. Evidence for Oxidative Stress

A variety of studies have provided evidence for a condition of oxidative stress in the parkinsonian brain.

Lipid peroxidation is a radical chain reaction evoked by exposure to hydroxyl radicals or other oxidants. Once initiated, it can be transmitted to adjacent lipids in membranes. The biological quenching agent is vitamin E. Two major changes are evoked by lipid peroxidation: The conjugation of previously separated double bonds in unsaturated lipids (such as linoleic, arachidonic, and linolenic acid) makes rigid previously flexible segments of membranes. Rigidity is related to the fact that rotation is restricted in conjugated dienes because the double bonds must lie in the same plane. The second change is the accumulation of fatty acid hydroperoxides, capable of altering membrane structure and function. Potentially toxic aldehyde products, such as 4-hydroxynonenal, are also formed. Dexter *et al.* (1989) reported an increase in lipid peroxides in the parkinsonian brain. The increase was associated with a simultaneous decrease in polyunsaturated fatty acids, and it was localized to the substantia nigra. Confirmatory evidence was provided by HPLC and electron spin resonance spectroscopy (Dexter *et al.*, 1994). Increased levels of 4-hydroxynonenal bound as an adduct to proteins have also been reported in PD (Yoritaka *et al.*, 1996). Jenner (1991) also reported elevated lipid peroxides in the parkinsonian brain.

A catalyst for lipid peroxidation is iron. Iron, specifically iron in the ferrous state, is also required for the production of hydroxyl radicals from H_2O_2 via the Fenton reaction [Eq. (3)]. A number of reports have described elevated tissue levels of iron in the parkinsonian brain and this has engendered strong interest in the exact location of the iron and whether or not it can contribute to the progression of PD (e.g., Olanow *et al.*, 1992).

The iron that can be visualized by histochemical stain in normal brain is relatively high in the substantia nigra, but it is restricted to the pars reticularis, whereas the DA cell bodies are present in another subregion, the pars compacta. However, the iron that accumulates in PD is seen in the pars compacta (Sofic *et al.*, 1991). The stainable iron is distributed in astrocytes, glia, microglia, and non-DA neurons (Jellinger *et al.*, 1990). However, analyses conducted by laser microprobe mass analysis (Good *et al.*, 1992) and X-ray microanalysis (Jellinger *et al.*, 1992) have revealed an accumulation associated directly with neuromelanin. So, a portion of the iron is localized within the melanized DA neurons.

Glutathione (GSH) in conjunction with GSH peroxidase normally detoxifies H_2O_2 and lipid peroxides within cells [Eqs. (8) and (9)]. GSH also reacts with and removes quinones derived from L-dopa. There is some evidence for decreased levels of GSH in the parkinsonian brain, which could expose DA neurons to the oxidant attack of peroxides. Sian *et al.* (1994) reported a mean 40% decline in GSH in the substantia nigra in 16 PD subjects, extending an earlier observation (Sofic *et al.*, 1991) based on only 4 PD subjects. The diminished GSH was not a secondary effect of nigral pathology as similar changes were not seen in Huntington's disease or progressive supranuclear palsy. However, the fact that lower GSH levels, as well as a change in the ratio of oxidized to reduced forms of glutathione, were seen in both PD and multiple system atrophy, both treated with L-dopa, has raised the question of whether these changes reflect drug therapy (Cohen, 1994).

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + 2H_2O \tag{8}$$

$$ROOH + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + ROH + H_2O \tag{9}$$

Direct oxidant damage to DNA (hydroxylation) has also been observed in the PD brain (Sanchez-Ramos *et al.*, 1994). In cell culture, DA neurons from rat brain are also susceptible to damage by environmental conditions or endogenous enzymatic activities: The number of surviving neurons can be increased two- to fourfold by adding antioxidants and/or simply lowering the oxygen tension (Colton *et al.*, 1995).

4. THEORIES ABOUT PARKINSON'S DISEASE

Theories about the etiology of PD and disease progression have centered on the research experience with the two neurotoxins, 6-OHDA and MPTP. One point of view is that oxy-radicals and peroxides, generated by natural metabolic pathways, such as MAO activity, promote a background of oxidative stress that spurs loss of DA neurons. This concept suggests a mechanism for disease progression, but does not explain etiology. A second point of view is that exposure to environmental toxins, perhaps related to MPTP and producing the same mitochondrial defect at the level of complex I, underlies both the development and progression of PD. This point of view is supported by the observation that PD is associated with a defect in mitochondrial Complex I (e.g., Schapira *et al.*, 1990; Mizuno *et al.*, 1995). Either theory may be affected by a genetic component that predisposes DA neurons to either oxidative stress or mitochondrial defects. In addition, the accumulation of iron must be considered. At the present time, iron fits better as a

catalyst for oxidative events (the MAO hypothesis) than it does for damage by environmental toxins (the MPTP hypothesis).

4.1. The MAO Hypothesis

The MAO hypothesis (Cohen, 1983, 1986) flows directly from the observations that H_2O_2 plays an essential role in the neurotoxic mechanism for 6-OHDA. If H_2O_2 is essential, a very prominent route for its production is via the oxidative deamination of DA by monoamine oxidase [Eq. (10)]. DA is a substrate for both MAO-A and MAO-B. The levels of DA in DA nerve terminals are quite high: An average concentration of 50 mM (Anden *et al.*, 1966) reflects a cytosolic pool in the range of 0.5–2.0 mM (e.g., Corrodi and Jonsson, 1967) and the larger amounts present in the vesicular storage pool.

$$DA + O_2 + H_2O \xrightarrow{MAO} \text{aldehyde} + H_2O_2 + NH_3 \qquad (10)$$

The surviving nigrostriatal neurons in PD show increased turnover of DA (Hornykiewicz and Kish, 1986). Similar observations have been made experimentally in rats after partial lesioning of nigrostriatal neurons by 6-OHDA, particularly when the lesion encompassed greater than 80% of DA neurons (Hefti *et al.*, 1980; Altar *et al.*, 1987). These observations are in keeping with the operation of a feedback regulatory loop between the striatum and the substantia nigra: As dopaminergic neurotransmission falls off as a result of nigral pathology, the system becomes disinhibited and increased firing rates, associated with increased release of DA, characterize the surviving DA neurons. A portion of released DA that is recaptured by the DA nerve terminal (uptake via the transporter) is oxidized by MAO. Some of the released DA is metabolized postsynaptically by catechol-*O*-methyltransferase and MAO. For each mole of DA or 3-*O*-methyl-DA oxidized by MAO, 1 mole of H_2O_2 is formed [Eq. (10)]. Considerable H_2O_2 can be generated as the 50 mM DA present within DA terminals is turned over both pre- and postsynaptically in the immediate vicinity of DA neurons. Therefore, an oxidant stress is directed at surviving DA neurons by enhanced MAO activity with DA as substrate; this effect is a natural consequence of the partial loss of DA neurons. In this sense, DA acts as an endogenous neurotoxin in PD.

4.2. The Environmental Toxin (MPTP-like) Hypothesis

The MPTP hypothesis is more self-evident. MPTP, or an agent like it, can gain access to brain and become transformed metabolically into a mitochondrial poison. There has been considerable interest in the possibility that exposure to herbicides or pesticides may promote parkinsonism (Tanner and Langston, 1990; Gorrell *et al.*, 1996). MPTP, via its metabolite MPP^+, poisons Complex I of the respiratory chain. The as yet unexplained presence of a mitochondrial defect in Complex I in PD (Mizuno *et al.*, 1989; Schapira *et al.*, 1990) may reflect exposure to environmental MPTP-like agents.

4.3. The Link between the MAO and MPTP Hypotheses

MAO is a constituent of the outer mitochondrial membrane, while Complex I and the other electron transport enzymes are present in the inner mitochondrial membrane.

An intermembrane space intervenes. Nonetheless, a new study (Cohen *et al.*, 1997) has demonstrated a linkage between MAO activity in the outer membrane and damage to electron transport at the inner membrane.

Exposure of rat brain mitochondria to 500 μM DA (or other monoamines) suppressed mitochondrial electron flow. The effect was significantly greater (56% inhibition) when the metabolic substrate was pyruvate, which initiates electron flow at Complex I, than with succinate (28% inhibition), which initiates electron flow at Complex II. Mitochondria were completely protected by MAO inhibitors. Mitochondrial damage was also reversed during electron flow. A probable explanation is that MAO-generated H_2O_2 oxidizes glutathione to glutathione disulfide [GSSG, Eq. (8)], which undergoes thiol-disulfide interchange with protein thiols (Pr-SH) to form protein mixed-disulfides [Pr-SSG, Eq. (11)] thereby interfering reversibly with thiol-dependent enzymatic function. In agreement with this interpretation, MAO activity induces mitochondrial loss of GSH (Sandri *et al.*, 1990), elevation in GSSG (Werner and Cohen, 1993), and accumulation of protein mixed-disulfides (Cohen *et al.*, 1997); these effects are also blocked by inhibition of MAO. Reversal during electron flow may be mediated by reduction of GSSG and Pr-SSG by reducing equivalents (NADPH, via transhydrogenase) generated during metabolism of pyruvate or succinate.

$$\text{GSSG} + \text{Pr-SH} \rightarrow \text{Pr-SSG} + \text{GSH} \tag{11}$$

It follows, therefore, that defects in mitochondrial respiration associated with PD may reflect, in part, the established increase in DA turnover. This means that the MAO and MPTP (mitochondrial poisoning) hypotheses are linked. The potential for mitochondrial damage may be enhanced when DA turnover is further sustained during chronic treatment with L-dopa (e.g., Przedborski *et al.*, 1993).

5. NEW DIRECTIONS IN PARKINSON RESEARCH

Several new areas related to oxidative stress and cell death are emerging in Parkinson research.

One report (Hunot *et al.*, 1997) described an increase in the proportion of DA neurons in the PD brain at autopsy with NF-κB translocated to the nucleus. NF-κB is a transcription factor that activates the expression of certain genes, including those responsible for several antioxidant enzymes, such as the biosynthesis of GSH and the mitochondrial (manganese) form of superoxide dismutase. Translocation of NF-κB can be caused by an oxidative stress. The implication is that NF-κB labeling of DA nuclei confirms the presence of an oxidative stress in PD. Translocation of NF-κB is also part of a signaling mechanism that induces programmed cell death (apoptosis).

A gene defect has also been described in a Parkinson-prone kindred of Italian descent (Polymeropoulos *et al.*, 1997). Although this is only one subgroup in the Parkinson spectrum, it offers an opportunity to identify factors that may be part of a common thread leading to the loss of nigrostriatal neurons. The gene defect in PD has been localized to a presynaptic protein, α-synuclein, which was previously implicated in Alzheimer's disease. An interesting aspect is that the gene "defect" in amino acid sequence in human subjects is the "normal" form of rodent synuclein. Therefore, it is an enigma that these

animals do not spontaneously show signs of parkinsonism during aging. Nonetheless, this genetic breakthrough opens opportunities for uncovering new details about the pathogenesis of PD.

A third new direction is really an old one. Does L-dopa carry a liability as well as a therapeutic benefit? This question still needs resolution. The therapeutic benefits of L-dopa are obvious and can be contrasted with experimental paradigms where damage is evoked, as well as the ever-present concern that indices of oxidant damage are directly affected by chronic treatment with L-dopa. To this turbulent mix, two new factors are added. One concerns the widespread belief that mitochondrial defects promote cellular demise, including apoptotic cell death. If true, then L-dopa exhibits a potential for promoting mitochondrial damage either directly (Przedborski *et al.*, 1993; Werner *et al.*, 1994) or indirectly via the metabolism of DA (Cohen *et al.*, 1997). The second factor concerns another widespread belief that peroxynitrite, formed in a reaction between superoxide and nitric oxide [Eq. (12)], can promote cellular damage and cell death:

$$\cdot O_2^- + \cdot NO \rightarrow ONOO^- \qquad (12)$$

Treatment with L-dopa elevates tissue DA levels, and tissue DA can scavenge superoxide; therefore, peroxynitrite formation will be impeded. Are these events relevant in either a negative way (mitochondrial damage) or a positive way (suppression of peroxynitrite) to the progression of PD?

Basic research on PD has opened vistas into the pathways of oxidative stress and their basic role in neurodegenerative processes. One continues to hope that the time is either now or soon at hand when this new information will open possibilities for clinical application to block the relentless and debilitating loss of DA neurons that characterizes PD. The DATATOP study (Parkinson Study Group, 1989a,b, 1993) was the opening sally into the realm of "antioxidant" therapy, an attempt to block disease progression at its roots. Although clinical success in the trial with deprenyl was modest and temporary (Parkinson Study Group, 1993; Olanow *et al.*, 1995), it has clearly opened a window into an approach that will certainly see further development and improved clinical success.

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